# Altering ruminant urine composition to reduce urine patch nitrous oxide emissions

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by

Camilla Allen Gardiner

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## Pre-Publication of Parts of this Thesis

Chapter 2 - a manuscript from this section has been published as follows:

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Chapter 3 - a manuscript from this section has been published as follows:

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Chapter 4 - a manuscript from this section has been published as follows:

Gardiner CA, Clough TJ, Cameron KC, Di HJ, Edwards GR, de Klein CAM 2017. Potential inhibition of urine patch nitrous oxide emissions by *Plantago lanceolata* and its metabolite aucubin. New Zealand Journal of Agricultural Research: doi: 10.1080/00288233.2017.1411953.

Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy

# Altering ruminant urine composition to reduce urine patch nitrous oxide emissions

by
Camilla Allen Gardiner

Grazed pasture systems are a significant source of nitrous oxide ($N_2O$), a potent greenhouse gas and ozone-depleting substance. In grazed pastures, $N_2O$ emissions are driven by ruminant livestock urine nitrogen (N) deposition onto soils. Urine N is typically deposited at a rate that exceeds the pasture plant's immediate N uptake capacity, therefore excess N may be lost from the system as $N_2O$. Two novel methods for mitigating urine patch $N_2O$ emissions were identified and evaluated in this PhD:

1. Altering urine N composition to increase the proportion of urine N excreted as non-urea urine nitrogen compounds (NUNCs). These NUNCs may be less labile forms of N, capable of stimulating plant N uptake, or forms of N that degrade to compounds which inhibit nitrification, a key step in soil $N_2O$ production.

2. Pasture species may contain active plant secondary metabolites (PSMs) capable of inhibiting nitrification in soil. After grazing ruminant livestock consume PSMs in their forage diet, the livestock would excrete them in their urine, thereby directly applying a nitrification inhibitor to the urine patch. The PSM, aucubin, in the pasture herb species *Plantago lanceolata* (plantain) was identified for its potential to inhibit nitrification in the urine patch.

In Chapter 3, the potential for varying urine N composition to alter urine patch $N_2O$ emissions was evaluated in a laboratory trial and a field trial. The laboratory trial tracked the fates of two $^{15}N$-labelled NUNCs in soil and the field trial determined the effect of increasing the proportion of urine N excreted as NUNCs, rather than as urea, on urine patch $N_2O$ emissions. In the laboratory trial, the $^{15}N$-labelled NUNCs, creatine and hypoxanthine, degraded in soil within 102 hours, and significantly contributed to both the soil N and plant N pools within 48 hours. In the field trial, increasing the proportion of urine N excreted as any of the NUNCs did not alter urine patch $N_2O$ emissions or any other measured variable. It was concluded that NUNCs rapidly degrade in soils and contribute to inorganic N pools that are substrates for urine patch $N_2O$ emissions, similarly to urea N.

Therefore, this proposed urine patch $N_2O$ mitigation technique is not viable and should not be further pursued.

In Chapter 4, both a plantain leaf extract (PLE) and an aucubin solution (AS) were applied with urine or urea (700 kg N $ha^{-1}$) under laboratory conditions, to mimic livestock excreting PSMs from plantain. This experiment determined whether plantain contained a PSM that could inhibit nitrification in the urine patch, and whether that PSM was aucubin. Overall average soil $N_2O$ flux was significantly lower in the Urea + PLE and Urea + AS treatments than in the Urine treatment. Additionally, soil nitrate ($NO_3^-$) concentrations on Day 29 were significantly lower in the Urea + AS, Urine + AS, and Urine + PLE treatments, when compared to the Urine treatment. These soil $NO_3^-$ and $N_2O$ flux results indicated that aucubin inhibited nitrification when applied to soil. A subsequent field trial was performed to evaluate the *in situ* effects of adding PLE or AS to urine (700 kg N $ha^{-1}$). Soil $N_2O$ emissions were lower in both the Urine + PLE and Urine + AS treatments, when compared to the Urine treatment, but the reduction was only statistically significant in the Urine + AS treatment. However, the lack of significant differences in the soil inorganic N data indicated that nitrification inhibition occurred. This field experiment also evaluated whether urine patch $N_2O$ flux or $NO_3^-$ accumulation were reduced in plantain pasture soil, due to the presence of aucubin released via root exudation, but no marked differences were observed.

Chapter 5 re-evaluated the *in situ* effects of adding aucubin to the urine patch, at a lower urine N application rate (500 kg N $ha^{-1}$), which has been identified as a more typical urine N loading rate from cows grazing plantain. Statistically significant differences in soil $NO_3^-$ concentrations and soil surface pH indicated that nitrification inhibition occurred 4-7 days after urine application, when aucubin was added in urine. However, this period of inhibitory activity was not sufficient to produce a significant reduction in $N_2O$ emissions over the 35 day experiment. It was concluded that this rate of aucubin application in urine was not sufficient to reduce urine patch $N_2O$ emissions.

In Chapter 6, three rates of aucubin application (47, 243, and 487 kg $ha^{-1}$) were added to urine and assessed for their potential to inhibit nitrification and subsequent $N_2O$ emissions. These rates represented 10, 50, and 100% of the highest calculated potential aucubin excretion rate from cows grazing 100% plantain pasture. Similar to the results reported in Chapters 4 and 5, there was a period from 7-17 days after urine application in the two higher aucubin treatments, where soil N and soil pH measurements indicated that aucubin inhibited nitrification. However, similar to Chapter 5, this period of activity was not sufficient to reduce overall $N_2O$ emissions during the experimental period.

It was concluded that further research of aucubin as a nitrification inhibitor was warranted, due to the periods of nitrification inhibition activity observed in the studies in Chapters 4-6. Further research is needed on: (i) the input rates and pathways of aucubin input into pasture soils via livestock urinary

excretion, plantain root exudation, and/or decomposition of residual herbage; (ii) the fate of aucubin in soil using isotope tracing methods; and (iii) molecular studies to identify the effect of aucubin on soil nitrifiers.

**Keywords:** nitrous oxide, ruminant urine, non-urea urine nitrogen, biological nitrification inhibition, aucubin

# Acknowledgements

Firstly, I would like to thank my main supervisor, Professor Tim Clough, for his guidance throughout this process. I am so grateful for all the time he spent discussing ideas for experiments, editing manuscripts or chapter drafts, and mentoring me as I progressed through this PhD program. Working with him made the daunting task of completing a PhD an enjoyable and rewarding experience.

I would also like to thank my co-supervisors, Keith Cameron, Hong Di, and Grant Edwards for their input and support. When I got caught up with the small details of my work, they encouraged me to keep the big picture in mind.

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The love and support from my family and friends back in the United States has meant so much to me over these years, even though they did not (and likely still do not) understand why I moved halfway across the world to study cow pee. It is difficult to live far away from people I love so much, but their endless encouragement and enthusiasm has taught me that no amount of distance can truly separate us.

To my loving partner, Tom, I cannot express to you enough how much I appreciate your support during these past three years. You have been there for me through all the ups and downs, and I am forever grateful for it.

Finally, I'd like to thank all my friends at Lincoln who made my experience so much fun. My wonderful officemates, fellow PhD students, and friends in the department made every day enjoyable.

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# List of Abbreviations

| | |
|---|---|
| AMO | Ammonia monooxygenase |
| AOA | Ammonia oxidising archaea |
| AOB | Ammonia oxidising bacteria |
| AS | Aucubin solution |
| BD | Bulk density |
| BNI | Biological nitrification inhibition |
| C | Carbon |
| $CO_2$-eq. | Carbon dioxide equivalents |
| CP | Crude protein |
| DCD | Dicyandiamide |
| DM | Dry matter |
| DOC | Dissolved organic carbon |
| $D_p/D_o$ | Soil gas diffusivity |
| EF | Emission factor |
| GHG | Greenhouse gas |
| HA | Hippuric acid |
| IG | Iridoid glycoside |
| ITC | Isothiocyanate |
| N | Nitrogen |
| $N_2O$ | Nitrous oxide |
| $NH_2OH$ | Hydroxylamine |
| $NH_3$ | Ammonia |
| $NO_2^-$ | Nitrite |
| $NO_3^-$ | Nitrate |
| NUNC | Non-urea urine nitrogen compound |
| $O_2$ | Oxygen |
| O1C4 | The field trial performed for Objective 1, Chapter 4 |
| PD | Purine derivative |
| PL | Plantain |
| PLE | Plantain leaf extract |
| PR-WC | Perennial ryegrass-white clover |
| PSM | Plant secondary metabolite |
| SEM | Standard error of the mean |
| WFPS | Water-filled pore space |
| WSC | Water soluble carbohydrate |

# Chapter 1
# Introduction

## 1.1 Background

Grazing livestock urine deposition onto pasture soil produces nitrous oxide ($N_2O$), a greenhouse gas and ozone-depleting substance (Ravishankara et al. 2009). It is known that urea, the dominant form of nitrogen (N) excreted in urine, is transformed into $N_2O$ in soils through microbially-mediated processes (Haynes & Williams 1993; Selbie et al. 2015). The development of management techniques to mitigate $N_2O$ emissions from urine patches is of particular importance in New Zealand, where urine patches are a dominant source of $N_2O$ emissions (Ministry for the Environment 2016). Previous research has identified that compounds which inhibit nitrification, a key process in transforming urine N to $N_2O$ in soils, can significantly reduce urine patch $N_2O$ emissions (Di & Cameron 2002a, 2012). However, issues with the implementation of these inhibitors, such as the potential transferral of inhibiting compounds into food products and the lack of suitable methods for directly targeting application onto urine patches, has stopped their wide-spread adoption (Di & Cameron 2016). Novel strategies for abatement of urine patch $N_2O$ emissions are required.

This PhD thesis assesses two novel methods for reducing urine patch $N_2O$ emissions: non-urea urine nitrogen compounds (NUNCs) and plant secondary metabolites (PSMs), with particular focus on aucubin, a PSM found in plantain (*Plantago lanceolata*). Both NUNCs and PSMs are excreted in urine, and their concentrations in urine can be altered through forage diet manipulation (Szanyiova et al. 1995; Keir et al. 2001; Totty et al. 2013). Therefore, these mitigation techniques do not require any chemical inputs, as they are based on forage diet manipulation, and will directly target the urine patch, since these compounds are excreted in urine.

The potential for NUNCs and/or aucubin to act as nitrification inhibitors is addressed in the literature review and the subsequent research chapters. Additionally, NUNC-N may be less labile in soils than urea-N, or NUNC compounds may stimulate plant N uptake. Therefore, increasing the proportion of N excreted as NUNC-N, rather than urea-N, may reduce substrate availability for $N_2O$-producing microbial processes and subsequently reduce $N_2O$ emissions.

## 1.2 Research Objectives

The objectives of this PhD research programme were to:

- Determine the fate of NUNCs in the urine patch and assess their contribution to urine patch $N_2O$ emissions;
- Evaluate whether increasing the proportion of urine N excreted as NUNC-N rather than as urea-N altered urine patch $N_2O$ emissions;
- Investigate whether plantain contained a PSM that acted as a nitrification inhibitor in urine patch conditions and if so, determine if aucubin was the active PSM;
- Examine the efficacy of the identified inhibitory PSM (aucubin) under field conditions; and
- Quantify the rate of the identified inhibitory PSM (aucubin) needed to significantly reduce urine patch $N_2O$ emissions.

## 1.3 Thesis Structure

This thesis is divided into 7 chapters. The first and second chapters provide an overview of the thesis topic and a review of the relevant literature, respectively. The next four chapters contain a short introduction and a materials and methods section, and then present and discuss the results of the experiments conducted. The final chapter summarises the overall findings of this thesis and provides directions for future research.

Chapter 1 This chapter gives a general overview of the topic of this thesis, the research objectives, and an outline of the thesis structure.

Chapter 2 This chapter summarises the background knowledge in a literature review and provides the reasoning and justification for the research conducted in this PhD thesis.

Chapter 3 This chapter presents one laboratory and one field experiment, which evaluated the fate of NUNCs in pasture soils and determined the effects of varying concentrations of NUNC-N on subsequent urine patch $N_2O$ emissions, respectively.

Chapter 4 A preliminary laboratory experiment and a field experiment are presented in this chapter. These experiments investigated whether plantain contained a PSM that inhibited nitrification, and whether aucubin was that active PSM.

Chapter 5 This chapter presents a field trial which continued to evaluate the *in situ* efficacy of aucubin as a nitrification inhibitor.

Chapter 6 This chapter summarises a laboratory experiment that quantified the aucubin application rate needed to produce significant reductions in $N_2O$ emissions from urine-affected soils.

Chapter 7 This chapter summarises the results from Chapters 3-6 and provides recommendations for future research on this topic.

# Chapter 2
# Literature Review

*A manuscript from this literature review has been published in the New Zealand Journal of Agricultural Research: Gardiner CA, Clough TJ, Cameron KC, Di HJ, Edwards GR, de Klein CAM 2016. Potential for forage diet manipulation in New Zealand pasture ecosystems to mitigate ruminant urine derived $N_2O$ emissions: a review. New Zealand Journal of Agricultural Research 59(3): 301-317. This is referred to in later chapters as Gardiner et al. (2016).*

## 2.1 Introduction

Nitrous oxide ($N_2O$) emissions account for over 10% of New Zealand's greenhouse gas emissions ($CO_2$-eq.; carbon dioxide equivalents) and are predominantly emitted from agricultural soils (Ministry for the Environment 2015a). Ruminant livestock urine N deposition is a driving factor for $N_2O$ losses from these soils. The objectives of this review are to:

- summarise the current knowledge on the impact of ruminant diet on grazed pasture N losses, and
- to analyse the potential ruminant urine patch $N_2O$ mitigation effects of non-urea urine nitrogen compounds (NUNCs), and plant secondary metabolites (PSMs).

Possible limitations to this approach for $N_2O$ mitigation and directions for future research are also discussed.

## 2.2 Significance of nitrogen in New Zealand agricultural systems

In New Zealand, agriculture is the predominant form of land use and contributes to over 65% of national exports (Statistics New Zealand 2013). Cattle and sheep, both ruminant species, are the most common livestock grazed in New Zealand, with approximately 30 million sheep, 6.7 million dairy cattle, and 3.7 million beef cattle raised on a total of 2.1 million hectares (Statistics New Zealand 2014). These ruminant livestock are primarily grazed on perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) (PR-WC) pasture systems, with supplementary winter feed supplied as required. Although clover supplies these pastures with nitrogen (N) through N fixation, substantial use is made of N fertilisers to maintain the productivity of these intensive systems (Gruber & Galloway 2008; Parfitt et al. 2008). In New Zealand, the use of urea fertiliser has increased five-fold since 1995 and currently exceeds 530,000 tonnes per year (Statistics New Zealand 2014). This fertiliser-N stimulates plant growth, but is redistributed within the grazed pasture ecosystem by the grazing animal, through consumption of plant N and subsequent excretion of urinary-N and

faecal-N, which may in turn result in the loss of N through numerous pathways, described below (Figure 2.1; Stark & Richards 2008; Sutton et al. 2011; Cameron et al. 2013).

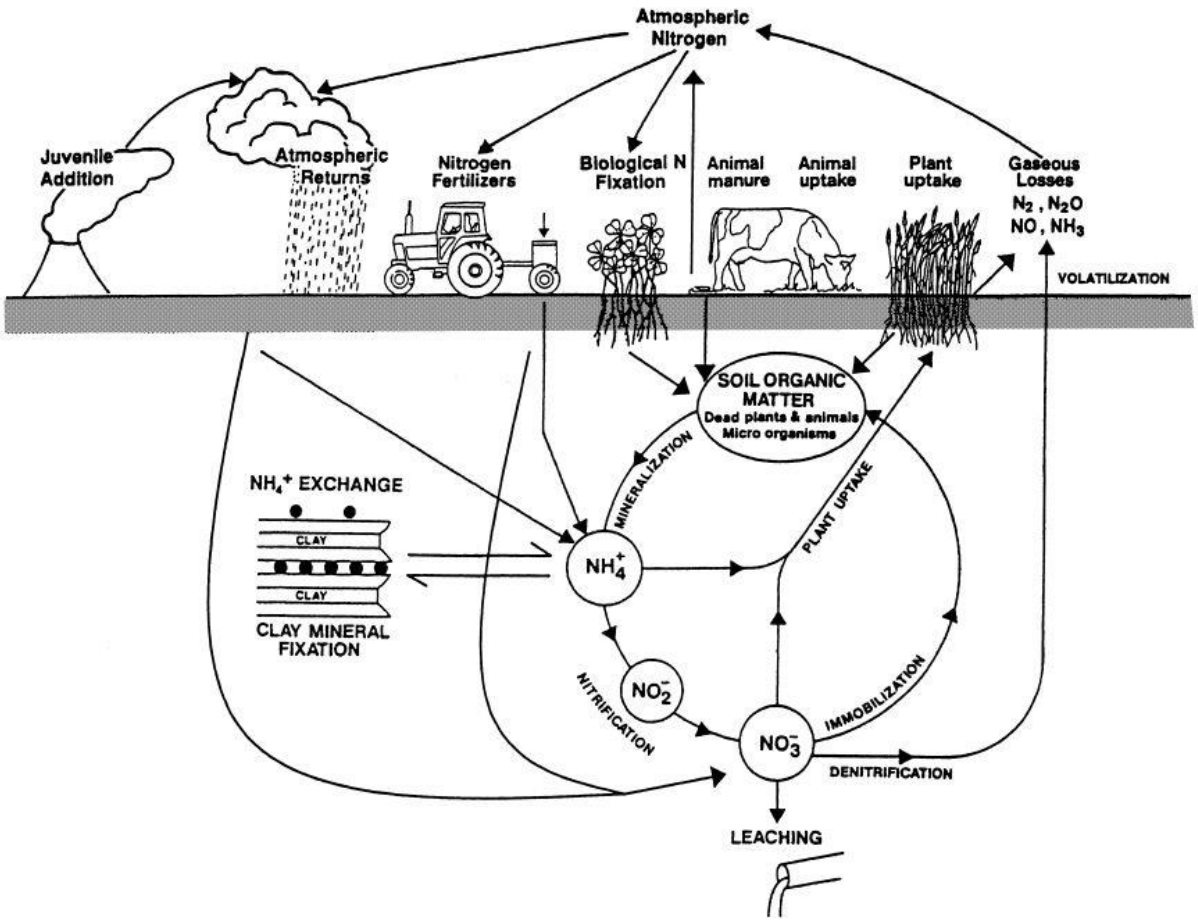



**Figure 2.1 Nitrogen cycling in agroecosystems (Di & Cameron 2002b).**

Ruminants are inefficient users of N and excrete 60-99% of their ingested dietary-N, with the percentage dependent on species type and dietary factors such as forage type or protein intake (Jarvis et al. 1995). Typically, this N is excreted predominantly as urinary-N, however, faecal-N can sometimes represent a higher proportion of N excretion when ruminants are fed a high protein diet (Barrow 1987; Jarvis et al. 1995; Dijkstra et al. 2013). As a result of high urine N concentrations, urination events apply more N to the soil than plants can immediately uptake, so losses of N are prevalent under urine patches (Haynes & Williams 1993). The most common forms of N lost from grazed pastures are nitrate ($NO_3^-$), ammonia ($NH_3$), $N_2O$ and dinitrogen ($N_2$) (Figure 2.1). Nitrate is lost via leaching while $NH_3$, $N_2O$, and $N_2$ are lost in gaseous forms. The gaseous loss of $N_2O$ is of particular importance because $N_2O$ is both a greenhouse gas (GHG) and an ozone-depleting substance (Ravishankara et al. 2009). New Zealand is a signatory to the Kyoto Protocol and must report on GHG emissions under both the United Nations Framework Convention on Climate Change and the Kyoto Protocol. The New Zealand government has a long-term GHG mitigation target of reducing total GHG

emissions to 50% below 1990 emission levels by 2050 (Ministry for the Environment 2015b). However, since 1990, there has been a 23% increase in New Zealand's agricultural $N_2O$ emissions, primarily due to an 88.4% increase in dairy cow numbers and a five-fold increase in fertiliser-N use (Ministry for the Environment 2015b). Nitrous oxide emissions of 9,050 kt $CO_2$-eq. accounted for 11.2% of New Zealand's total GHG emissions in 2013 with the largest source being agricultural soils, which were responsible for 93.4% of national $N_2O$ emissions (Ministry for the Environment 2015a). Emissions of $N_2O$ are calculated and reported to the IPCC using an emission factor (EF), which is the percent of applied N, or N added to a system, that is lost as $N_2O$. Urinary-N contributes significantly more to these emissions than faecal-N, with an estimated 23.2 Gg $N_2O$ $yr^{-1}$ (EF = 1%) emitted from urine N compared to just 2.7 Gg $N_2O$ $yr^{-1}$ (EF = 0.25%) emitted from faecal-N (Luo & Kelliher 2010). Thus there is a strong need to develop management techniques to reduce $N_2O$ emissions from grazed pasture systems, particularly focussed on urine patches due to their larger $N_2O$ losses.

## 2.3 Urine patch N dynamics in grazed pastures

### 2.3.1 Urine volume, frequency, and N content

Urination volumes have been reported to average 1.6–2.2 L for dairy cattle and 0.10–0.18 L for sheep (Haynes & Williams 1993). Urination volumes vary significantly with the time of day and between individual animals, with variation generally correlated to differences in water and dietary mineral intakes (Betteridge et al. 1986; Eriksson & Rustas 2014). Cattle and sheep average 8–12 and 18–20 urinations per day, respectively, with the size of urine patch areas ranging from 0.16–0.49 $m^2$ and 0.03–0.05 $m^2$, respectively (n = 32) (Haynes & Williams 1993). The pasture area subsequently affected by urine deposition can be up to 3.4 times larger than the area directly wetted by urine (Lotero et al. 1966; Moir et al. 2011; Buckthought et al. 2016). Herd animals tend to spend time in concentrated groups, therefore resulting in excreta being returned to pasture in an uneven manner. Using a negative binomial distribution model, it was estimated that a typical New Zealand dairy farm would have 20–30% of its land covered by excreta in one year (Moir et al. 2011).

Ruminant urine N concentrations have been reported to range from 1–20 g N $L^{-1}$, averaging 6.9 g N $L^{-1}$ for dairy cattle and 8.7 g N $L^{-1}$ for sheep (Whitehead 1995; Dijkstra et al. 2013). This variation is due to many of the same factors that dictate urine volume, which are stated above. Nitrogen loading under dairy cattle urine patches can reach up to 1,000 kg N $ha^{-1}$, while sheep urine patches have lower N loading rates due to their smaller urination volume (Haynes & Williams 1993; Jarvis et al. 1995). These N rates exceed the pasture's immediate ability to uptake this deposited urinary-N, which typically ranges from 300–700 kg N $ha^{-1}$ $yr^{-1}$, therefore losses of N from the pasture system, as

described above, are common (Moir et al. 2007). The form of N excreted, either urine or dung, determines the magnitude and form of subsequent N losses.

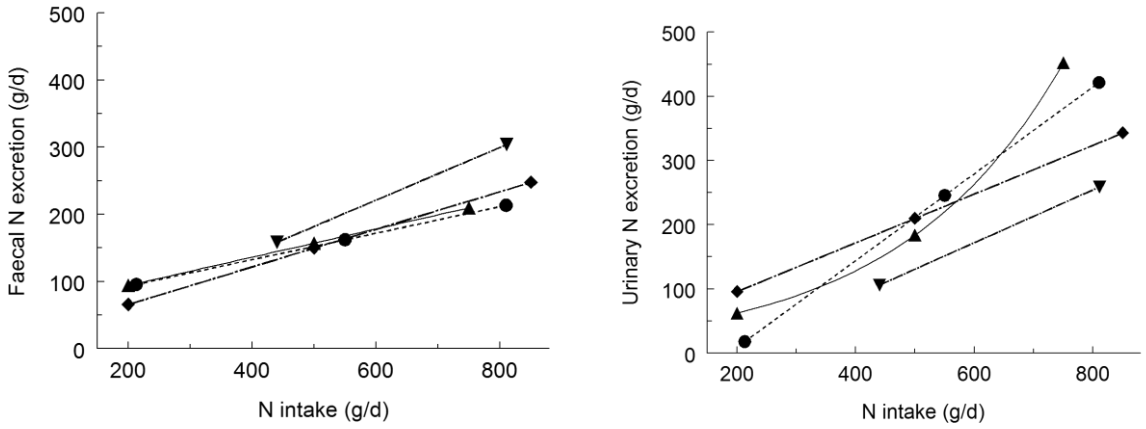



**Figure 2.2 Relationship between N intake (g N $d^{-1}$) and N excretion in urine (g N $d^{-1}$; left graph) and N excretion in faeces (g N $d^{-1}$; right graph) from Dijkstra et al. (2013), who compiled several studies (as referenced therein). ▲ Castillot et al. (2000); ▼ Weiss et al. (2009); ● Huhtanen et al. (2008); ♦ (Kebreab et al. 2010).**

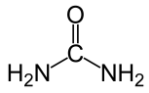
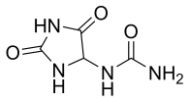
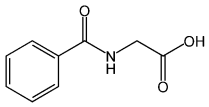
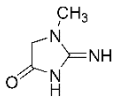
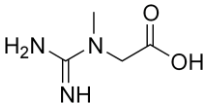
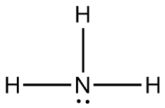
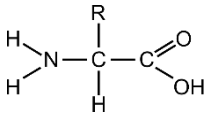
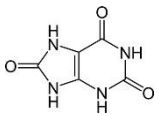
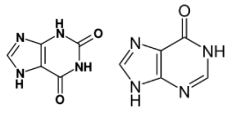
The proportion of N excreted as urine or faeces depends on animal species, dry matter intake, N content of the diet, and other dietary factors. Regardless of this complexity, there is a strong linear correlation between ruminant N intake and N subsequently excreted in either urine or dung: as N intake increases (g $d^{-1}$), the resulting increase in urinary-N (0.38–0.68 g $d^{-1}$) is greater than for faecal-N (0.20–0.39 g $d^{-1}$) (Figure 2.2). There is also a 3.5 times higher variation in urinary-N than faecal-N excretion (Dijkstra et al. 2013). Given the higher proportion of N excreted as urine, and the greater variation in the amount of urinary-N excreted per unit of N intake between studies, there is an increased potential to influence urine N output rather than faecal-N output through dietary manipulation.

### 2.3.2 Urine composition

Urinary-N originates within the rumen, where an imbalance between the degradation of pasture N substrates and the uptake of N by microbes leads to an excess of $NH_3$. The liver detoxifies the system by converting $NH_3$ into urea which is then excreted (Dijkstra et al. 2013). Within the digestive system, 9–81% of urea-N may be recycled via reabsorption into the gut through the portal drained viscera (Lapierre et al. 2005). Reducing dietary N intake can improve N use efficiency and reduce excretal N loading, however, diets too low in N can also be detrimental and impair growth (Kebreab et al. 2001;

Law et al. 2009). Nitrogen is predominantly excreted as urea, comprising 50–90% of the urinary-N excreted, with concentrations dependent on N intake. Urinary-N is also excreted as allantoin, hippuric acid, creatinine, creatine, ammonia, amino acids, uric acid, xanthine, and hypoxanthine (Table 2.1; Selbie et al. 2015). Improving knowledge and understanding of these non-urea urinary-N compounds (NUNCs) is one of the main aims of this PhD research.

**Table 2.1. Nitrogenous constituents of cattle urine (adapted from reviews by Dijkstra et al. (2013) and Selbie et al. (2015)).**

| Urinary Constituent | Chemical Structure | Average Concentration (g N $L^{-1}$) | Concentration Range (g N $L^{-1}$) | Average % of Total N |
|---|---|---|---|---|
| **Total N** | | 8.2 | 1.0–20.5 | – |
| **Urea** |  | 6.0 | 2.1–19.2 | 73 |
| **Allantoin** |  | 0.86 | 0.27–1.5 | 10 |
| **Hippuric acid** |  | 0.51 | 0.37–0.70 | 6 |
| **Creatinine** |  | 0.26 | 0.08–0.65 | 3 |
| **Creatine** |  | 0.26 | 0.12–0.51 | 3 |
| **Ammonia** |  | 0.20 | 0.03–1.0 | 2.5 |
| **Amino acids** |  | 0.15 | 0.03–0.3 | 2 |
| **Uric acid** |  | 0.08 | 0.03–0.18 | 1 |
| **(Hypo)xanthine** |  | 0.05 | 0.03–0.09 | 0.6 |

Hypoxanthine, xanthine, uric acid, and allantoin are derived from purine bases. These purine derivatives (PDs) are excreted as a consequence of microbial protein synthesis and are absorbed from the gastrointestinal tract or from other endogenous origins (Chen & Ørskov 2004). Purine bases are deaminated into hypoxanthine and xanthine, then rapidly converted to uric acid due to the high activity of xanthine oxidase in the liver and gastrointestinal tract of cattle. Uric acid is then oxidised to allantoin, which is the most readily excreted PD (Tas & Susenbeth 2007).

Allantoin is the only PD that consistently responds to variation in exogenous purine influx (Balcells et al. 1991). This is likely because additional protein increases rumen microbial growth, therefore increasing allantoin output (Blaxter & Martin 1962; Ramgaokar et al. 2008). Hence, urinary allantoin is used as an indicator of microbial protein synthesis in the rumen because it varies in concentration as a result of varying rumen nucleic acid concentrations (Dijkstra et al. 2013).

Creatine is produced in the kidney and liver and is used in muscle function (Wyss & Kaddurah-Daouk 2000). Creatinine is a product of the degradation of creatine or its phosphorylated creatine-phosphate form (Borsook & Dubnoff 1947). Creatinine is therefore a function of muscle metabolism, rather than dietary intake, and urinary concentrations can be used as a measure of muscle mass (Dijkstra et al. 2013).

The precursor compounds to hippuric acid (HA) are produced when phenolic acids are fermented in the rumen, and thus HA excretion is related to the dietary concentration of degradable phenolic acids (Martin 1982). Excretion of HA is positively correlated with dietary crude protein (CP) and negatively correlated with the lignin content of the diet, due to the decreased solubility and degradability of phenolic acids in these forms (Pazur & Delong 1948; Kehraus et al. 2006).

### 2.3.3 Pathways of nitrogen cycling in urine affected soil

Pasture uptake and soil immobilisation of urinary-N account for an average 41% and 26%, respectively, while losses via $NO_3^-$ leaching, $NH_3$ volatilisation, and $N_2O$ emissions average 18% (16–24%), 13% (1–38%), and 2% (0–14%), respectively (range in brackets) (Selbie et al. 2015). Few studies have directly measured $N_2$, so the range of reported losses, 30–65%, is quite wide (Monaghan & Barraclough 1993).

#### *2.3.3.1 Ammonia volatilisation*

Urea is the dominant N form found in ruminant urine and, once deposited onto the soil, it hydrolyses, creating an excess of $NH_4^+$ and carbonate ions (Equation 2.1; Sherlock & Goh 1984).

**Equation 2.1:** $CO(NH_2)_2 + 2H_2O \rightarrow 2NH_4^+ + CO_3^{2-}$

The carbonate ions then undergo hydrolysis (Equation 2.2), resulting in localised areas of increased pH, with increases as high as 2.5-3.5 pH units common in the topsoil within 24 hours of urine deposition (Sherlock & Goh 1984; Haynes & Williams 1992).

**Equation 2.2:** $CO_3^{2-} + H_2O \rightarrow HCO_3^- + OH^-$

The high pH causes $NH_4^+$ to dissociate to $NH_3$, which is then prone to volatilisation (Sherlock & Goh 1984). The volatilisation of $NH_3$ peaks 1–2 days after urine deposition with the amount of N lost highly variable, reportedly ranging from 1–38% of urine N applied (Selbie et al. 2015). However, a review of $NH_3$ volatilisation indicated that 10% is typical for New Zealand conditions (Sherlock et al. 2008). Soils with high cation exchange capacity will volatise less $NH_3$ because they have a higher retentive capacity for $NH_4^+$ (Haynes & Williams 1993). Ammonia volatilisation is not necessarily a complete loss because 20–60% of $NH_3$ can be redeposited within 2 m of the emission site, which may subsequently lead to plant uptake or further indirect losses of N via $NO_3^-$ leaching and $N_2O$ emissions (Ross & Jarvis 2001). However, such data for New Zealand pasture systems is lacking.

#### *2.3.3.2 Pasture uptake, soil immobilisation, and soil adsorption*

Any $NH_4^+$ not converted to $NH_3$, and volatised, may be taken up by plants, nitrified to $NO_3^-$, adsorbed onto mineral surfaces, or immobilised (Figure 2.1). The mineral N forms, $NH_4^+$ and $NO_3^-$, are taken up via plant roots, thus stimulating pasture growth. Pasture N uptake ranges from 300–700 kg N $ha^{-1}$ $yr^{-1}$ (Moir et al. 2007). Urinary-N, once converted to mineral N, can also be consumed by microbes and incorporated into soil organic matter, thereby immobilising it in the soil (Haynes & Williams 1993).

#### *2.3.3.3 Nitrate leaching*

Plant roots dominate the top soil, and the movement of mineral N below the plant rooting depth is considered a loss. Such movement occurs primarily as $NO_3^-$ leaching, but N can also be leached as $NH_4^+$ or urea. Various factors influence the degree of $NO_3^-$ leaching under urine patches, including the degree of plant N uptake, the magnitude of prior $NH_3$ loss, soil texture, soil tillage, pasture plant composition, timing of urine application, and inputs of irrigation or rainfall (Haynes & Williams 1993; Stark et al. 2007; Fraser et al. 2010; Fraser et al. 2013). Urine volume and urine N concentration also

contribute to the quantity of $NO_3^-$ leached. Sheep excrete smaller volumes of urine per urination event, therefore plants can take up a greater proportion of applied N and less N is lost through leaching. Thus, decreasing urine volume and/or urine N loading rate are potential techniques to reduce leaching losses (Ledgard et al. 2007; Cameron et al. 2013). In livestock systems, 70-90% of leached N originates from the urine patch (Ledgard et al. 2009).

#### *2.3.3.4 Nitrous oxide emissions*

Nitrous oxide is primarily produced through biologically mediated processes: nitrification, nitrifier-denitrification, and denitrification (Figure 2.3; Firestone & Davidson 1989; Wrage et al. 2001; Zhu et al. 2013). Nitrification is a two-step process carried out by chemoautotrophic nitrifying bacteria, first oxidising $NH_4^+$ to nitrite ($NO_2^-$) and then oxidising $NO_2^-$ to $NO_3^-$ (Firestone & Davidson 1989). Nitrification produces $N_2O$ from the chemical decomposition of hydroxylamine ($NH_2OH$) or the reduction of $NO_2^-$ via nitrifier-denitrification (Figure 2.3; Wrage et al. 2001). Nitrification is a net-acidifying process, so soil pH ultimately returns to its pre-urine deposition level following its urine-induced alkaline state (Haynes & Williams 1993). Nitrification rates are primarily controlled by $O_2$ and $NH_4^+$ availability, and high pH or high $NH_3$ concentrations can be inhibitory (Monaghan & Barraclough 1992; Venterea et al. 2015). Ammonia toxicity leads to a build-up of $NO_2^-$, and since $N_2O$ can be produced from $NO_2^-$, such accumulation leads to increased $N_2O$ emissions (Venterea et al. 2015). With warm soil temperatures nitrification can be complete within 30 days, but this process can take up to 60 days under low soil temperatures (Holland & During 1977).

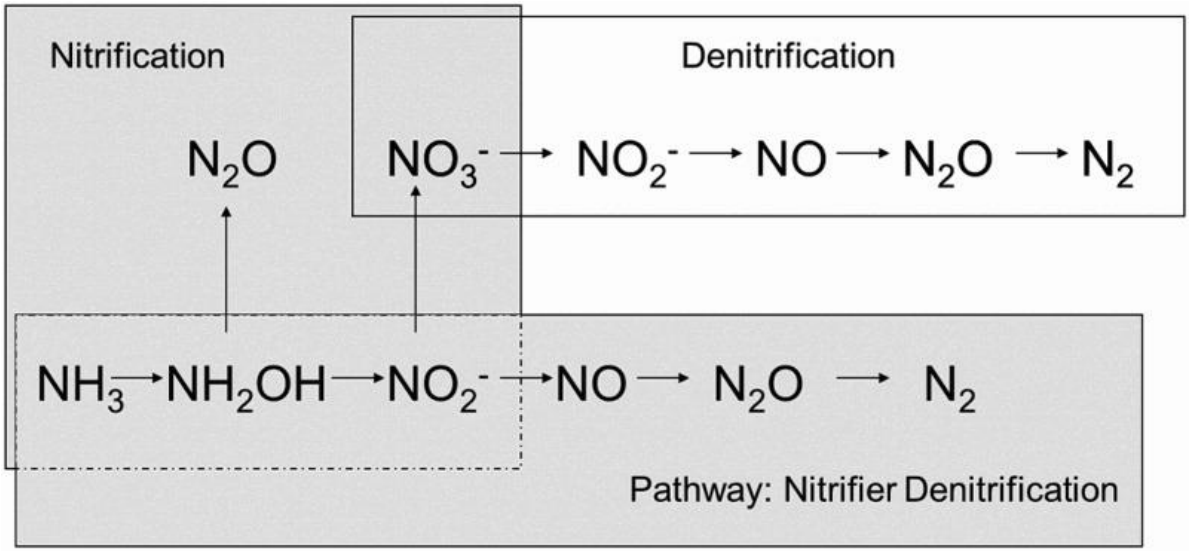



**Figure 2.3 Microbial inorganic N transformation pathways in soil (Wrage et al. 2001).**

Denitrifying organisms reduce $NO_3^-$ to $N_2O$ and $N_2$ (Figure 2.3). Denitrification is a heterotrophic process that is promoted under anaerobic conditions when substrate availability ($NO_3^-$ and organic carbon) is high. Urine patches are thus prime sites for denitrification because they can potentially provide all of these conditions (Haynes & Williams 1993). Soil water-filled pore space (WFPS) has been promoted as a predictor of conditions suitable for production of $N_2O$, with emissions peaking at 60–70% WFPS (van Groenigen et al. 2005b). However, relative soil gas diffusivity ($D_p/D_o$, where $D_p$ is the soil–gas diffusion coefficient ($cm^3$ soil air $cm^{-1}$ soil $sec^{-1}$) and $D_o$ is the gas diffusion coefficient in free air ($cm^2$ air $sec^{-1}$)) has been shown to be a stronger predictor of $N_2O$ emissions as it accounts for the interaction of soil bulk density and moisture content (Balaine et al. 2013). Emissions of $N_2O$ are generally higher during the rainy season because soils are wetter (reduced $D_p/D_o$) which enhances denitrification. However, low winter temperatures may slow bacterial activity (Haynes & Williams 1993). Peaks in $N_2O$ emissions immediately after urine application result from: the stimulation of denitrification if antecedent soil $NO_3^-$ is present to act as a substrate (Sherlock & Goh 1984); the creation of anoxic conditions following urea hydrolysis (Owens et al. 2016); and the time required for $N_2O$ reductase to be produced (Hansen et al. 2014). Soil $N_2O$ emissions could be mitigated by decreasing nitrification and denitrification rates, or increasing the ratio of $N_2$:$N_2O$ by promoting complete denitrification (de Klein et al. 2001; Di & Cameron 2003; Cameron et al. 2013).

## 2.4 Previously identified strategies for reducing urine patch $N_2O$ emissions

Urine patch $N_2O$ emissions are a function of two factors: the total urine patch N loading and the percent of applied N that is emitted as $N_2O$, known as the emission factor (EF) (Luo et al. 2008). The urine EF could be reduced by either lowering the proportion of N applied in a form available for transformation, therefore reducing substrate availability, or directly inhibiting $N_2O$ production processes.

### 2.4.1 Reducing urine patch N loading via dietary manipulation

One of the main drivers for $N_2O$ loss from the urine patch is $NO_3^-$ availability, which is influenced by urine N loading (Firestone & Davidson 1989; de Klein et al. 2001). It is known that urine patch $N_2O$ emissions increase with increasing urine N loading (de Klein et al. 2001; van Groenigen et al. 2005b; Selbie et al. 2014). Altering forage diet composition to reduce urine N content by redistributing N to other excretal pathways, such as milk and faecal excretion, has been identified as a method to lower urine patch $N_2O$ emissions (Totty et al. 2013; Edwards et al. 2015; Box et al. 2016; Cai et al. 2017).

#### *2.4.1.1 Common and diverse forage types in New Zealand*

The most common pasture forage type in New Zealand is a combination of perennial ryegrass and white clover (PR-WC). Ryegrasses have high water-soluble carbohydrates (WSCs), and provide a good source of energy for microbial activity in the rumen and hindgut (Edwards et al. 2007). Tetraploid and diploid varieties contain different water contents, which affects urine volume and N loading rates (Pacheco et al. 2010). Clovers are N-fixing legumes and provide high yields of high nutrient value feed without need for N fertilisers (Steinshamn 2008).

Other forages commonly used in New Zealand are kale (*Brassica oleracea*), fodder beet (*Beta vulgaris*), chicory (*Cichorium intybus*), plantain (*Plantago lanceolata*), lucerne (*Medicago sativa*), maize *(Zea mays*), Italian ryegrass (*Lolium multiflorum*), and red clover (*Trifolium pratense*). Kale is used for its fast growth rates, high nutrient value, and cold tolerance (Belesky et al. 2007). Fodder beet is also highly cold tolerant and has a higher dry matter (DM) and WSC content than kale (DairyNZ 2013). Chicory and plantain are both perennial herbs. Chicory is highly palatable and highly productive, but can become winter dormant and may taint milk flavour if consumed at over 50% of the diet. Plantain grows well in pastures with high disturbance, but can be out competed by perennial ryegrass and weeds (Belesky et al. 2007). Lucerne is a perennial legume that can fix N and has a long tap root, making it both nutrient dense and drought resistant (Palmer & Wynn-Williams 1976). Maize provides a dense source of carbohydrates and is mainly used as a silage addition to grazed diets in winter when forage productivity can be low (DairyNZ 2008).

#### *2.4.1.2 Previous research on the effects of forage diet on urine N excretion*

While N intake is a key factor in determining N excretion rates, variation in dietary carbohydrate and CP content can also significantly alter rumen N use efficiency, which determines the quantity of N retained or excreted (Bach et al. 2005; Dijkstra et al. 2013). Reducing CP in ruminant diets generally leads to decreases in urinary-N excretion (Topps & Elliott 1967; Spek et al. 2013). This is likely due to decreased protein degradation in the rumen, which reduces $NH_3$ accumulation, and increases renal absorption of urea (Bach et al. 2005).

Increasing dietary carbohydrate intake has also been shown to increase N excretion in faeces by 76%, most likely as endogenous N (N embodied within microbes and microbial products from the rumen, intestine and hind gut, plus digestive tract N), because post-ruminal microbial synthesis is greatly dependent on energy availability (Dijkstra et al. 2013; Spek et al. 2013). Low-degradability carbohydrates are more likely to pass through the rumen into the hind-gut and stimulate microbial activity, therefore increasing faecal-N output (Kebreab et al. 2001). Faecal-N is less susceptible to short-term loss, and the $N_2O$ emission factor for faecal-N (0.25%) is 25% that of urinary-N (1%) (Luo &

Kelliher 2010). Therefore, increased N excretion as faecal-N rather than urine N potentially reduces overall $N_2O$ emissions. However, if dietary changes increase the endogenous N content of the faeces, there is likely to be an increase in faecal $N_2O$ emissions (Jost et al. 2013). There is also the potential for increased $CH_4$ emissions with increased carbohydrate consumption, but this would easily be offset by the resulting reduced $N_2O$ emissions (Dijkstra et al. 2013).

Totty et al. (2013) found that a diverse diet of chicory, plantain, lotus (*Lotus pedunculatus)*, high sugar ryegrass, and white clover led to a 17–19% decrease in urinary-N content compared to PR-WC diets. The diverse diet had a lower CP content but a higher WSC content, likely leading to higher rumen N use efficiency (Totty et al. 2013). This diet also resulted in an increase in milk urea-N, which supports the hypothesis that increasing the ratio of WSC:CP in diets produces more milk urea-N and less urine-N (Edwards et al. 2007).

A recent study by Box et al. 2016 found that cows grazing 100% plantain diets had urine N concentrations significantly (>50%) lower than cows grazing PR-WC pasture. Interestingly, faecal-N concentrations between the two diets were not different, and milk urea-N concentrations were lower from cows grazing 100% plantain diets. The reduction in urine N may have been due to either an increased WSC:CP intake ratio, or an increased mineral intake leading to increased urine volume (Ledgard et al. 2015; Box et al. 2016). Similarly, Cheng et al. (2017), Judson and Edwards (2016), and Minneé et al. (2017) found that cows feeding on plantain, or whose diets were supplemented with plantain, had significantly lower urine N concentrations compared to cows feeding on PR-WC or dietary supplements of pure perennial ryegrass. The average urine N loading rate is reduced from 700 kg N $ha^{-1}$ to approximately 500 kg N $ha^{-1}$ when plantain is incorporated into the diet of grazing cows (Di et al. 2016).

Urine N loading can also be reduced by incorporating salt or mineral supplementation to increase water consumption, thereby diluting N excretion with an increased total urination volume and frequency (Ledgard et al. 2007; Dijkstra et al. 2013; Ledgard et al. 2015). Two studies have found a diuretic effect when plantain was fed to sheep or cows, compared to PR-WC, even though the animals on both diets had the same water consumption (O'Connell et al. 2016; Cheng et al. 2017). However, increasing urine volume does not consistently reduce urine patch $N_2O$ emissions, possibly due to increased WFPS in the urine patch, which would enhance denitrification rates (Dijkstra et al. 2013).

### 2.4.2 Synthetic nitrification inhibitors

A number of synthetic compounds, most notably dicyandiamide (DCD), have been shown to reduce $N_2O$ emissions from soils with varying effect (Di & Cameron 2003; Carey et al. 2012; Dennis et al. 2012; Li et al. 2013; Cameron et al. 2014; de Klein et al. 2014a; Kim et al. 2014; Ledgard et al. 2014; Ruser & Schulz 2015; Cai et al. 2017). Synthetic inhibitors have mainly focussed on ammonia oxidising bacteria (AOB), which perform the first step in nitrification (Di & Cameron 2003). It has been shown that AOB, rather than ammonia oxidising archaea (AOA), are the main drivers of nitrification in soils, with the activity of AOB increasing 177-fold after the application of an ammonia substrate, while AOA showed no response in activity levels (Di et al. 2009).

Inhibiting AOB subsequently decreases $NO_2^-$ and $NO_3^-$ production, both of which directly lead to $N_2O$ emissions. A summary of published data (140 data sets) indicated that synthetic inhibitors on average could potentially reduce $N_2O$ emissions by approximately 35% (Ruser & Schulz 2015). Issues with the implementation of synthetic inhibitors include the lack of suitable methods for directly targeting urine patches, increased input costs for the farmer, potential transferral of inhibiting compounds into food products (e.g. milk), verifying the efficacy of inhibitors in situ, and variations in efficacy due to soil type, drainage, and climate (Balaine et al. 2013; Cameron et al. 2014; de Klein et al. 2014a; Kim et al. 2014; Ledgard et al. 2014).

## 2.5 Novel strategies for reducing urine patch $N_2O$ emissions

There is no current mitigation method implemented in New Zealand based on either of the above strategies for reducing urine patch $N_2O$ emissions, due to either a lack of applicable management methods or issues with implementation (e.g. transfer of the chemical into milk). Therefore, there is a need to find novel mitigation techniques. Diverse forage pastures, pastures with a large number of species or pastures that are predominantly comprised of species other than perennial ryegrass and white clover, have the potential to lower the urine EF by increasing the proportion of urine N excreted as NUNCs and/or by increasing the urinary excretion of plant secondary metabolites (PSMs) (Clough et al. 2009; Dietz et al. 2013). This is because the N found in NUNCs may be less labile, thereby reducing N substrate availability, while PSMs could potentially inhibit $N_2O$-producing processes.

### 2.5.1 Excretion of urine N as non-urea urine nitrogen compounds

Urea typically constitutes 73% of urine N and the remaining 27% is excreted as NUNCs (Selbie et al. 2015). While the degradation of urea in the urine patch has been well studied, it remains unknown

how NUNCs degrade in urine-affected soils and to what extent they contribute to the urine patch $N_2O$ emission factor. If NUNCs were less labile in soils than urea, then manipulating grazing ruminant livestock's diets to increase the proportion of urine N excreted as NUNCs could be an effective mitigation technique to reduce urine patch $N_2O$ emissions.

#### *2.5.1.1 Dietary influence on urine N composition*

Dietary management can strongly influence the content and concentration of N in urine, as explained previously, and therefore how much N is ultimately available for soil nitrification and denitrification processes. Since New Zealand ruminant livestock are raised primarily on forage, the potential for dietary manipulation comes mainly from altering forage types. Diverse forage diets may also have other effects on excretal properties that contribute to GHG emissions, such as urine pH or excretal carbon (C) partitioning, but these effects will not be addressed in this review.

While the decline in total urinary-N loading is predominantly due to reduced urea excretion, one study showed the concentrations of all NUNCs, with the exception of uric acid, declined when ruminants were fed low protein diets (Szanyiova et al. 1995). Additionally, Box et al. (2016) showed that increasing plantain in dairy cow diets decreased total urine N concentration by reducing both urea and creatinine concentrations. Urinary-N composition can also be affected by N intake, with diets high in N increasing the proportion of urinary-N excreted as urea (Topps & Elliott 1967; Jarvis et al. 1995; Selbie et al. 2015). The range of reported concentrations of NUNCs in urine is presented in Table 2.1. It is known that varying forage diet alters urine N composition, but it remains unknown how the resulting changes in urine N composition alters the urine patch $N_2O$ EF. If increased excretion of urinary-N as NUNCs leads to a reduced urine patch $N_2O$ EF, then grazing strategies could be developed to increase urinary-N excretion of those NUNCs.

#### *2.5.1.2 Degradation of NUNCs in soil*

##### 2.5.1.2.1 Catabolism of purine derivatives in soil

Ruminant animals consume excess amounts of N and must convert N into excretal forms to avoid $NH_3$ toxicity. These excretal forms, as noted above, include the PDs: hypoxanthine, xanthine, uric acid, and allantoin (Table 2.1). In addition, many plants, especially legumes, contain significant concentrations of allantoin and allantoic acid, which are important components in the storage and movement of plant N (Wang et al. 2007). Therefore, deposition of plant litter also contributes to soil PD inputs.

However, since N is frequently a limiting nutrient for plants and soil microbes, plants and microbes have developed many enzymatic pathways to catabolise purine compounds and access the embodied N for new growth (Figure 2.4; Vogels & van der Drift 1976; Schultz et al. 2001; Werner & Witte 2011). As a consequence, the ability to degrade purine compounds can be found in all kingdoms and occurs under both aerobic and anaerobic conditions (Vogels & van der Drift 1976).

The oxidation of hypoxanthine and xanthine is driven by the enzyme xanthine dehydrogenase, found in soil bacteria such as *Pseudomonas aeruginosa,* and which results in the formation of uric acid (Figure 2.4; Vogels & van der Drift 1976) . The uricase enzyme, an oxido-reductase enzyme, converts uric acid to allantoin and is also found widely within the soil environment (Vogels & van der Drift 1976).

Allantoin has two optical isomers which may undergo racemisation (Figure 2.4). Racemases catalyse the stereochemical inversion of optical isomers and allantoin racemase has been found in *Pseudomonas* species along with allantoinase, which allows the organisms to use both isomer forms of allantoin. Allantoinase, the enzyme degrading allantoin, is very common in the environment, occurring in animals, higher plants and microorganisms (Vogels & van der Drift 1976). There appears to be very few studies that have examined the fate of allantoin in soil, however, in rice paddy soils allantoin has been shown to be released from rice plant roots with subsequent increases in both microbial biomass and diversity (Wang et al. 2007; Wang et al. 2010). The half-life of allantoin was reported to be 20.2 ± 2.5 hours and 7.3 ± 1.9 hours for autoclaved and non-autoclaved soils, respectively, indicating rapid biodegradation or transformation (Wang et al. 2007).

Under strongly alkaline conditions allantoin may form allantoate which is, in turn, degraded by the enzymes allantoicase and allantoate amidohydrolase. The latter catalyses allantoate into $NH_3$ and carbon dioxide, while allantoicase catalyses allantoate into urea and glyoxalate (Figure 2.4; Vogels & van der Drift 1976). Urea in soil will be hydrolysed (Equation 2.1) and potentially contribute to $N_2O$ production.

Allantoin has been shown to have a stimulatory effect on plant growth (Shetty et al. 1992; Pan et al. 1994; Wang et al. 2007; Sun et al. 2012). Stimulation of plant growth in the urine patch would increase plant N uptake, and therefore reduce N substrate availability for $N_2O$ emissions. It is possible that this effect would reduce urine patch $N_2O$ emissions. Since the other PDs (hypoxanthine, xanthine, and uric acid) degrade to allantoin, it is possible that all PDs could stimulate plant N uptake. Therefore, manipulating forage diets to promote the excretion of urine N as PDs rather than as urea could possibly reduce the urine patch EF.

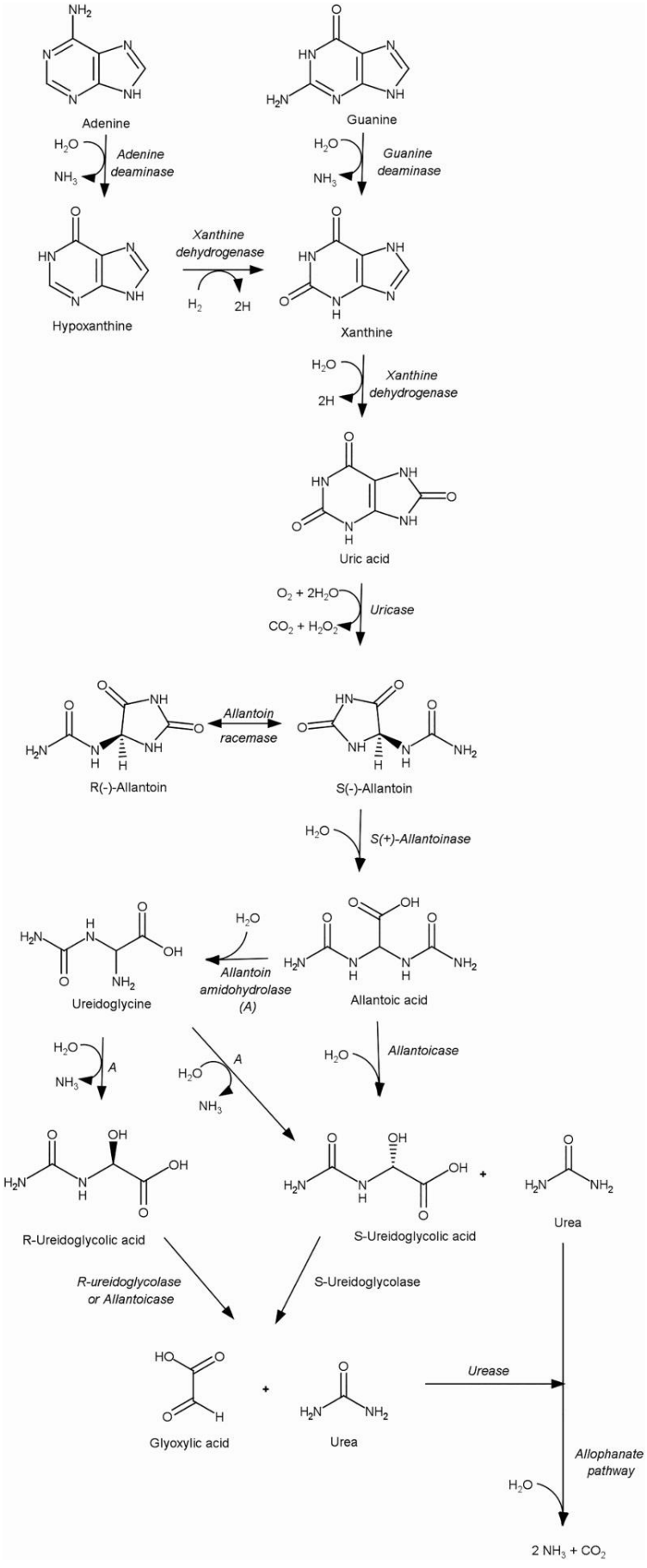



**Figure 2.4 Pathways of purine degradation in aerobic soils, from Vogels and van der Drift (1976).**

#### 2.5.1.2.2 Catabolism of creatine, creatinine, and hippuric acid in soil

Degradation of creatinine can occur through two pathways, with either creatine or N-methylhydantoin as the first product (Shimizu et al. 1986). The metabolic pathway via creatine is well established and results in the production of urea and sarcosine, an intermediate in glycine synthesis (Kopper 1947; Kopper & Robin 1950; Kaplan & Szabo 1974; Yoshimoto et al. 1976; Rikitake et al. 1979). The degradation pathway via N-methylhydantoin predominantly results in the accumulation of N-methylhydantoin as the only degradation product, but numerous microbial strains can metabolise N-methylhydantoin to glycine, an amino acid that is quickly taken up by plant roots (Kim et al. 1986; Shimizu et al. 1986; McFarland et al. 2010). Creatinine was found to disappear within 4–5 days from soil samples incubated aerobically at 30°C and 37°C under both acidic and neutral conditions. Under anaerobic conditions or high temperature (59°C), creatinine was still present after 2 weeks (Dubos & Miller 1937). Consequently, it is unlikely that variations in urine creatinine or creatine concentrations will have any impact on urine patch $N_2O$ emissions, due to their degradation to urea or other microbially-available substrates.

Hippuric acid is a conjugate compound of glycine and benzoic acid (Bristow et al. 1992). There is no detailed information on the degradation of HA in soils, but it is most likely hydrolysed to glycine and benzoic acid, with the subsequent production of $NH_4^+$ resulting from the deamination of glycine (Bristow et al. 1992). Benzoic acid has been examined for its effects on $N_2O$ emissions, as discussed below.

#### 2.5.1.2.3 Previous studies on the effect of NUNCs on $N_2O$ emitting processes

Of the NUNCs listed in Table 2.1, only HA has been examined as a potential mitigation tool for soil $N_2O$ emissions. HA and its derivative benzoic acid, a known antimicrobial agent, reduced $N_2O$ emissions in lab experiments, but failed to produce significant results in situ (Kool et al. 2006a; Bertram et al. 2009; Clough et al. 2009; Krol et al. 2015). Laboratory studies showed a 48–65% decrease in $N_2O$ emissions with increased HA content of the urine, most likely due to the inhibition of denitrification or a decrease in the $N_2O$:$N_2$ product ratio (Kool et al. 2006a). The non-significant results in situ were potentially due to loss of HA through macropore flow, high degradability of benzoic acid in alkaline conditions, or the response of in situ microbial communities differing to in vitro conditions (Clough et al. 2009). However, any potential effects of the other NUNCs on $N_2O$ emissions have yet to be ascertained.

### 2.5.2 Plant secondary metabolites as biological nitrification inhibitors

Recent studies have quantified urine patch $N_2O$ emissions on a variety of pasture types used in New Zealand, with some pasture types producing significantly lower $N_2O$ emissions than others (Di et al. 2016; Byrnes et al. 2017; Luo et al. 2018). Di et al. (2016) did not find a significant reduction of $N_2O$ emissions after urine deposition on diverse pasture (including chicory, plantain, perennial ryegrass, and white clover), fodder beet, kale, lucerne, or Italian ryegrass, when compared to a PR-WC pasture. However, Luo et al. (2018) found a significant reduction in $N_2O$ emissions after urine deposition on plantain pasture compared to PR-WC pasture in three out of four urine deposition trials. It was also shown that urine patch $N_2O$ emissions were reduced on forage grass species that are known to inhibit the activity of soil nitrifiers, known as biological nitrification inhibition (BNI) (Byrnes et al. 2017). It is possible that the reduction of urine patch $N_2O$ emissions on plantain pasture found in Luo et al. (2018) could be due to BNI activity in plantain.

The hypothesised mechanism of BNI activity is the inhibition of nitrification by allelo-chemicals produced by plants that enter soils through root exudation or addition to soils via plant leaf litter (Lata et al. 1999; Subbarao et al. 2007). Plants produce a variety of secondary metabolites that do not act towards primary growth or development, but contribute to plant fitness through antimicrobial and allelopathic activity (Bourgaud et al. 2001). Nitrification could be inhibited by these plant secondary metabolites (PSMs) acting directly on enzymatic processes to suppress microbial activity (Subbarao et al. 2007; Baggs et al. 2010).

If a pasture forage species was identified to have BNI activity, incorporating this species into grazed pasture could reduce pasture N loss through two methods: 1. releasing inhibitory PSMs into pasture soil through root exudation or, 2. producing inhibitory PSMs in their leaf matter, which would then be consumed and excreted by grazing livestock, therefore directly applying the inhibitory PSM into the urine patch.

#### *2.5.2.1 Previous research on biological nitrification inhibition*

Biological nitrification inhibition has been studied in forested and grassland soils, with the most success reported under grassland systems (Rice & Pancholy 1974; McCarty & Bremner 1986; Subbarao et al. 2007). Some plants that have been identified to produce BNI chemicals include *Sorghum bicolor*, *Brachiaria humidicola*, and *Oryza sativa* (Coskun et al. 2017). Some of the identified BNI compounds include sorgoleone (*S. bicolor*), brachialactone (*B. humidicola*), caffeic acid (e.g. *Pinus ponderosa*), ferulic acid (e.g. *Pinus echinata*), and linoleic acid (*B. humidicola*) (Coskun et al. 2017). These compounds can block or inhibit ammonia monooxygenase (AMO), the enzyme which regulates the first step of nitrification, through three different mechanisms: direct binding and interaction with

AMO, interference with the reductant supply to AMO, or oxidation of substrate to produce compounds that inactivate and/or are toxic to AMO (Subbarao et al. 2006). These advances in BNI research have been proposed as a method to increase nitrogen use efficiency in crop and pasture production, either by growing plants with high BNI activity or by genetically modifying non-BNI plants to include BNI genes (Rosolem et al. 2017; Subbarao et al. 2017).

Denitrification has also been shown to be inhibited by PSMs (Bardon et al. 2014). However, focussing on AMO inhibition could lead to greater $N_2O$ reductions because such inhibition reduces the production of most precursors to $N_2O$-producing pathways (Wrage et al. 2001). However, biological inhibition of nitrification and/or denitrification has not been widely tested in grazing systems. There is considerable potential for BNI activity to significantly reduce N loss from grazed pasture soils, either resulting from N fertiliser application or urine deposition.

#### *2.5.2.2 Studies to date under urine patch conditions*

One study on this topic to date showed a significant reduction in $N_2O$ emissions when sheep were grazed on *Brassicas*, which are known to contain BNIs (Luo et al. 2015). *Brassicas* contain isothiocyanates (ITCs), a product of glucosinolate hydrolysis (Figure 2.5A,B). ITCs are found only in *Brassicas* and are toxic to microbes because they irreversibly change the tertiary structure of enzymes, therefore inhibiting metabolic processes (Brown & Morra 1997). ITCs can be released into the soil from roots, but also could enter the soil through plant matter degradation and animal excreta (Choesin & Boerner 1991). In an incubation study, ITCs were shown to inhibit AOB in both sandy- and clay-loam soils, showing their versatility as inhibitors under different soil conditions (Bending & Lincoln 2000).

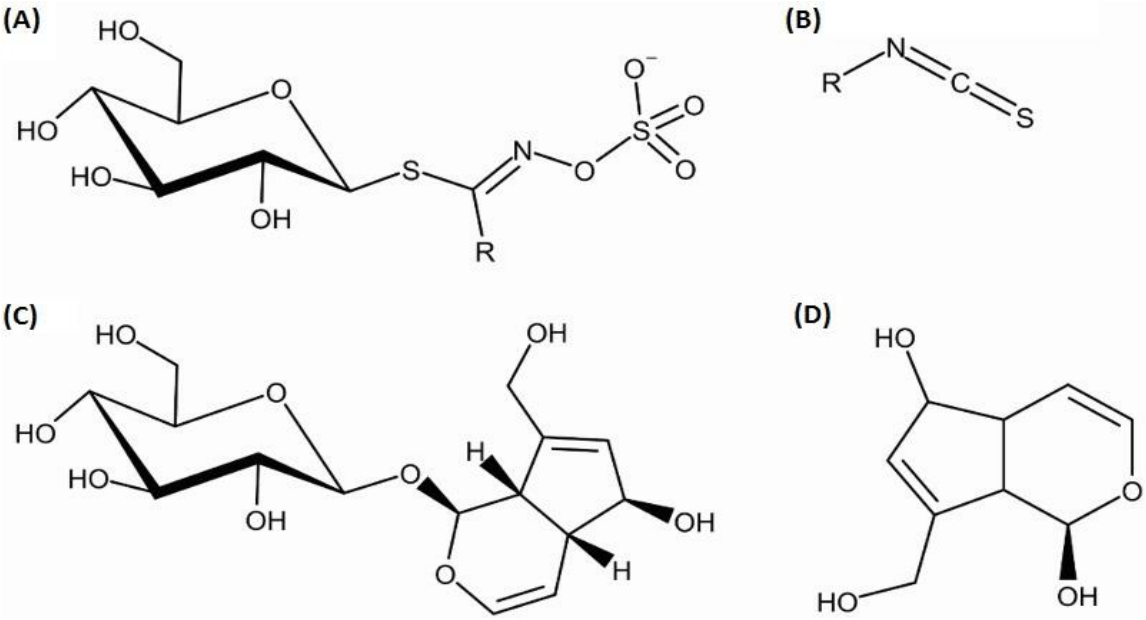



**Figure 2.5 The chemical structures of glucosinolates (A), ITCs (B), aucubin (C), and aucubigenin (D). Glucosinolates and ITCs vary in structure depending on the substituted R-group.**

However, further analysis of ITCs showed that only one of five ITCs reduced soil $N_2O$ emissions under laboratory conditions, but none of the tested ITCs reduced soil $N_2O$ emissions under field conditions (Balvert et al. 2017). Results from this study showed that the inhibition by ITCs was short-lived and would likely require multiple applications if used as a nitrification inhibitor. Therefore, ITCs do not seem to be a viable nitrification inhibition option for use in New Zealand.

### *2.5.2.3 Aucubin as a nitrification inhibitor*

Aucubin (Figure 2.5C), an iridoid glycoside (IG) found in plantain, has recently been found to inhibit nitrification in soil (Massaccesi et al. 2015). Soils under plantain pasture were reported to have lower $NO_3^-$ concentrations, mineralisation and nitrification rates than under other plants species, with increasing rates of mineralisation and nitrification as plantain abundance decreased (Massaccesi et al. 2015). Similarly, a decline in soil N mineralisation was found when potato was undersown with plantain (Rauber et al. 2008). In a laboratory incubation study, applying plantain leaf extracts to soil resulted in almost complete inhibition of $NO_3^-$ production for 56 days when the extract from 3 g of plant matter was added (Figure 2.6; Dietz et al. 2013). Another study found that the activity and populations of nitrifying bacteria were reduced up to 200 fold in the presence of plantain plants, however, it was concluded that this reduction was not a result of inhibitory PSMs (Verhagen et al. 1995).

Inhibitory activity likely comes from aucubigenin, an aglycone of aucubin, which is known to have antimicrobial activity (Figure 2.5D; Figure 2.6A,B; Ishiguro et al. 1982; Ishiguro et al. 1983; van der Sluis et al. 1983; Davini et al. 1986; Bartholomaeus & Ahokas 1995). Aucubigenin is produced when the glucose ring in aucubin is hydrolysed by β-glucosidase (Davini et al. 1986). Ammonia oxidation is inhibited by chemicals that interact with cytochrome P-450, a hemeprotein complex, and aucubigenin is known to inhibit this particular protein structure (Bartholomaeus & Ahokas 1995). Aucubin has shown characteristics of competitive and non-competitive inhibition, and could inhibit enzyme activity through direct binding and interaction with AMO through protein cross-linking via Schiff reactions (Bartholomaeus & Ahokas 1995; Subbarao et al. 2006). Aucubigenin can irreversibly inhibit enzymes by reacting its dialdehyde tautomer with free amino groups (Davini et al. 1986; Bartholomaeus & Ahokas 1995; Kim et al. 2000). The reactivity of the dialdehyde structure of aucubigenin can lead to the formation of a very wide variety of products (Ghisalberti 1998; Migneault et al. 2004).

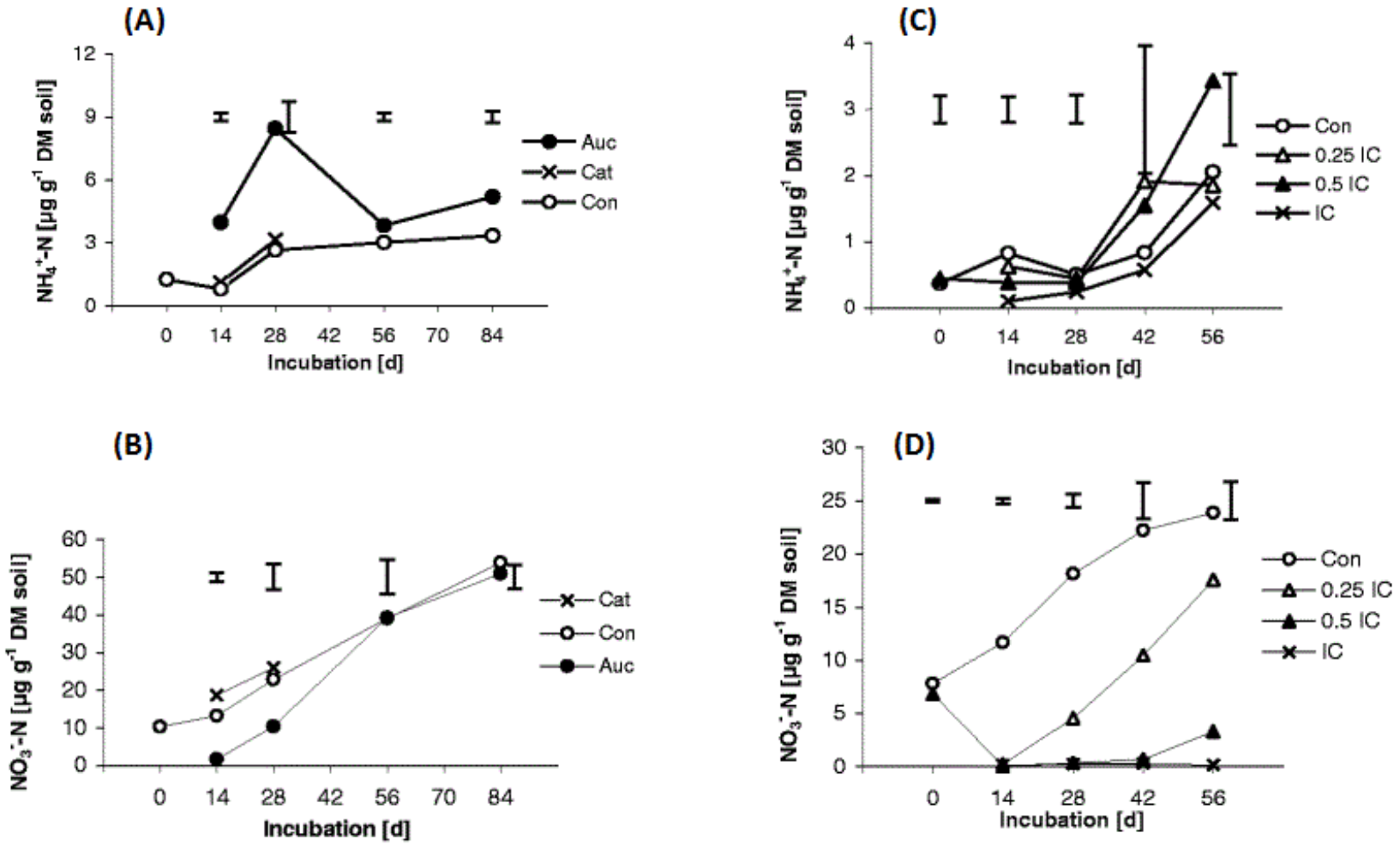



**Figure 2.6 Soil $NH_4^+$-N (A and C) and $NO_3^-$-N (B and D) during a laboratory incubation experiment, when either aucubin or catalpol (two IGs in plantain; A and B) or a plantain leaf extract (C and D) were applied to soils (Dietz et al. 2013). 'IC' represents an extraction of 3 g plantain leaf matter, and '0.5 IC' and '0.25 IC' are 0.5 and 0.25 dilutions of this extract.**

**Figure 2.7 A photo taken in October 2016 of plantain growing at the Lincoln University Research Dairy Farm, showing the leaves and scapes (flowering stems).**

Plantain produces IGs as herbivore and pathogen deterrents, and it is known that aboveground herbivore and pathogen activity, as well as belowground soil microorganism and nematode concentrations, can induce the production of IGs in plantain (Bowers & Puttick 1988; Darrow & Bowers 1999; Marak et al. 2002; Fuchs & Bowers 2004; Wurst et al. 2010). Aucubin is present in plant leaves (1-5%), scapes (1%), and roots (1-2%), with concentrations varying between species and over time (reported range % DM in brackets) (Adler et al. 1995; Darrow & Bowers 1999; Tamura & Nishibe 2002). There is no decrease in plant performance with increased concentrations of defensive chemicals, which shows that there is no trade-off between plant defence and plant growth (Adler et al. 1995). Bioactive compounds in plantain may have beneficial medicinal value for grazing animals, and have been shown to: reduce liver damage in mice (Chang et al. 1983; Yang et al. 1983; Chang et al. 1984); increase the kidney function of lambs (Deaker et al. 1994b); increase sheep tissue responsiveness to insulin (Sano et al. 2003); reduce inflammation (Recio et al. 1994); and protect against tumours (Ishiguro et al. 1986). However, aucubin was found to be toxic to mice at rates about

200 mg $kg^{-1}$ (Ishiguro et al. 1986). A rumen fluid study found that plantain herbage slows rumen microflora activity, but does not impair rumen function (Deaker et al. 1994a). A further in vitro rumen fermentation experiment showed a 40% reduction in $NH_3$ production over 24 hours when plantain was added to rumen fluid, compared to chicory, indicating that plantain could reduce ruminant urinary-N excretion (Navarrete et al. 2016). It is suggested that rumen microbes degrade aucubin and can tolerate the antimicrobial effects of aucubigenin (Navarrete et al. 2016).

Out of 550 plant species surveyed, plantain was the only species with antimicrobial properties that was detectable in rabbit urine 8–16 hours after feeding (Stewart 1996). A rabbit is a monogastric animal and not a ruminant but it is likely that similar results may occur for ruminant urine. Goat urine has also been analysed to determine whether urinary PSM excretion could be used as a marker to estimate diet composition (Keir et al. 2001). Metabolites were only present in urine for 48 hours after feeding on plant material, but the pattern of metabolite excretion varied according to the species of plant ingested, showing that there is potential for aucubin to be excreted in dairy cattle urine as a result of plantain forage diets.

Plantain has been evaluated as a forage species for ruminant livestock production in New Zealand in many grazing studies (Totty et al. 2013; Box et al. 2016; Judson & Edwards 2016; O'Connell et al. 2016; Cheng et al. 2017; Minneé et al. 2017). These studies have shown that plantain can be fed to livestock at up to 100% of their dietary composition with no negative effect to animal health or production. Furthermore, Minneé et al. (2017) and Box et al. (2016) show that incorporating plantain into dairy cattle diets reduces urine N concentrations, while maintaining or increasing milk production. Additionally, it has been shown that incorporating plantain into PR-WC pasture increases pasture DM production (Moorhead & Piggot 2009). Therefore, evaluating plantain for its potential to reduce urine patch $N_2O$ emissions is sensible, as this pasture species has been shown to be successful in New Zealand livestock production systems and to be beneficial to the management of N losses from these systems.

#### *2.5.2.4 Other plants with possible inhibitory PSM activity*

Some other PSMs have also shown cytochrome P-450 interactions, but these have not been assessed with respect to nitrification and $N_2O$ emission processes in soil. For example, furanocoumarins, commonly found in grapefruits (*Citrus paradisi*) or spring parsley (*Petroselinum crispum*), are known to inhibit cytochrome P-450, and these feeds could potentially be added as a dietary supplement (Guo & Yamazoe 2004; Abivardi 2008).

Endophyte, a fungal infection, common in ryegrasses, contains many known active metabolites that could also be evaluated for their impact on N transformations. Janthitrems, an endophytic mycotoxin, has been isolated from ruminant dung, and it is an example of an endophyte PSM that demonstrates passage through the ruminant digestive system without degradation (di Menna et al. 2012). Thus, other classes of PSMs, such as flavonoids or tannins, have been used as antimicrobial medicine for centuries and could also be assessed with respect to their potential to inhibit nitrification or denitrification (Cushnie & Lamb 2005).

#### *2.5.2.5 Potential limitations of using PSMs as nitrification inhibitors*

It is also important to consider the potential negative side effects of incorporating such biologically active compounds into ruminant diets. For example, furanocoumarins significantly increase cattle photosensitisation and over consumption could lead to skin blistering (Abivardi 2008). While PSMs have the potential to inhibit microbes associated with nitrification, there is the possibility that other microbes in the soil or rumen could be inhibited, thus potentially leading to unexpected or unwanted side effects. PSMs could also potentially become present in milk, therefore exposing consumers to heightened levels of these active compounds. Future research thus needs to evaluate the potential for diets containing cytochrome P-450-inhibiting or other antimicrobial compounds to inhibit soil $N_2O$ emitting processes, while bearing in mind the potential ramifications for ruminant and human health.

## 2.6 Summary

Diet significantly affects ruminant N output, altering both total N excretion rate and the form in which N is excreted. Ruminant urine N composition is also altered under varying diets (Topps & Elliott 1967; Szanyiova et al. 1995; Selbie et al. 2015). However, it remains unknown how varying urine N composition, particularly the increased excretion of NUNCs, alters the urine patch $N_2O$ EF. One NUNC, HA, has been evaluated for its potential to reduce $N_2O$ emissions, but the effects of other NUNCs have not been quantified (Kool et al. 2006a; van Groenigen et al. 2006; Clough et al. 2009). Based on the literature, it is hypothesised that an increased excretion of urine N as PDs will stimulate plant growth, thereby increasing plant N uptake and reducing N substrate availability for $N_2O$ emissions, while increased excretion of urine N as creatine or creatinine will have no effect on the $N_2O$ EF. Future experiments need to be performed to confirm this hypothesis by showing that $N_2O$ emissions are not reduced by altering urine N composition and/or concentration, using $^{15}N$-labelled NUNCs to track their residence time and fate in urine patches.

Other future studies should focus on the promising field of PSMs. Aucubin, a PSM in plantain, has been identified as potential inhibitory compound for reducing urine patch $N_2O$ emissions. However, the inhibitory capacity of aucubin has not been tested under urine patch conditions. Aucubin could enter soils either through root exudation from plantain growing in pastures, or via urinary excretion from ruminants grazing plantain. Studies to quantify the residence time of aucubin in soil and its effect on urine patch $N_2O$ emissions and $NO_3^-$ are urgently needed. Further work should determine the rate of aucubin needed to significantly reduce urine patch $N_2O$ emissions. If aucubin shows potential as a nitrification inhibitor, pasture and grazing management practices could be developed to reduce the detrimental environmental effects of $NO_3^-$ accumulation in pasture soils and $N_2O$ emissions from ruminant urine patches.

This literature review identifies three key knowledge gaps/questions where there is a lack of research and understanding:

i. How do NUNCs contribute to urine patch $N_2O$ emissions and how does the composition of urine N affect the $N_2O$ EF?

ii. What is the potential for aucubin, a PSM in plantain, to reduce soil nitrification and subsequent $N_2O$ emissions under urine patch conditions?

iii. What rate of aucubin is needed to produce a significant reduction in urine patch $N_2O$ emissions?

The following research chapters will address these knowledge gaps/questions.

# Chapter 3
# Non-urea ruminant urine nitrogen compounds: assessing their fate in a pasture soil and their impact on the urine patch nitrous oxide emission factor

*A manuscript from these studies has been published in the Journal of Environmental Quality: Gardiner CA, Clough TJ, Cameron KC, Di HJ, Edwards GR, de Klein CAM 2018. Assessing the impact of non-urea ruminant urine nitrogen compounds on urine patch nitrous oxide emissions. Journal of Environmental Quality 47:812-819.*

## 3.1 Introduction

In New Zealand, agricultural soils account for 94% of national $N_2O$ emissions, due to the large extent of grazed pasture (Ministry for the Environment 2016). Ruminant urine nitrogen (N) deposition results in $N_2O$ emissions that, in total, are ca. 9-fold higher than those arising from faecal-N depositions. These urine patch $N_2O$ emissions are calculated and reported to the IPCC based on an emission factor (EF), which is the percent of applied urine N that is subsequently emitted as $N_2O$ (Luo & Kelliher 2010).

On average, 73% of ruminant urine N is excreted as urea, which is known to degrade to ammonium ($NH_4^+$) and nitrate ($NO_3^-$) in soil and can lead to losses of $N_2O$ (Selbie et al. 2015). It is unknown how the other forms of N present in ruminant urine, excreted as non-urea urine N compounds (NUNCs), contribute to urine patch $N_2O$ emissions or influence the urine patch EF (Gardiner et al. 2016).

One NUNC, hippuric acid (HA), was hypothesised to reduce urine patch $N_2O$ emissions by inhibiting ammonia oxidation, the first step in nitrification and a key process in soil $N_2O$ production, due to its breakdown product, benzoic acid, being a known antimicrobial compound (Marwan & Nagel 1986). However, benzoic acid's efficacy as a nitrification inhibitor has been inconsistent (Kool et al. 2006a; van Groenigen et al. 2006; Clough et al. 2009; Krol et al. 2015). Since the effects of HA on the $N_2O$ EF have already been studied, this research focusses on five other NUNCs: allantoin (10%), creatinine (3%), creatine (3%), uric acid (1%), and (hypo)xanthine (0.6%), where numbers in brackets represent the average percentage of total urine N (Selbie et al. 2015). Urinary excretion of NUNCs could reduce the urine patch EF if they, or their degradation products, inhibited nitrification, similar to the hypothesised effects of HA. Furthermore, if NUNCs are less labile in soils compared to urea, or degraded to compounds that are not readily used by $N_2O$-producing microbes, then increasing the proportion of urine N excreted as NUNCs could decrease the available substrate for $N_2O$ production and subsequently reduce urine patch $N_2O$ emissions.

The three NUNCs allantoin, uric acid, and (hypo)xanthine are purine derivatives (PDs), and can be degraded and used as N sources by many microorganisms, including algae, fungi, and bacteria (Vogels & van der Drift 1976). (Hypo)xanthine is degraded to uric acid and subsequently to allantoin, which has a reported half-life of 7.3 hours in rice paddy soil, indicating rapid microbial breakdown and/or plant uptake (Vogels & van der Drift 1976; Wang et al. 2007). Allantoin has also been shown to have a stimulatory effect on plant growth (Shetty et al. 1992; Pan et al. 1994; Wang et al. 2007; Sun et al. 2012). Stimulation of plant growth would potentially increase plant uptake of the urine N pool, thereby reducing the total available substrate for $N_2O$ emissions and decreasing the urine patch EF. As these compounds are readily degraded by microbes, it is unlikely that any of the PDs, or their degradation products, would inhibit nitrification, a microbially-mediated process.

The two other NUNCs of focus, creatine and creatinine, can be degraded in the soil through multiple pathways, resulting in the production of sarcosine (an intermediate in glycine synthesis), glycine, and urea (Kopper & Robin 1950; Kaplan & Szabo 1974; Shimizu et al. 1986). Creatinine was shown to disappear from aerobic soils within 4-5 d, under both acidic and neutral conditions (Dubos & Miller 1937). Therefore, increasing the proportion of urine N excreted as creatine or creatinine, rather than excreted as urea, would likely not reduce N availability for $N_2O$-producing processes, as these compounds rapidly degrade to microbially-available substrates. Additionally, a mixture of urea and glycine was shown to have similar $N_2O$ emissions to real urine (Kool et al. 2006b). Furthermore, it is unlikely that creatine or creatinine would act as a nitrification inhibitor, due to their rapid breakdown in soils to products that do not exhibit antimicrobial activity.

The objectives of this study were to assess the fate of NUNCs in the urine patch and determine the impact of NUNCs on the urine patch EF. A laboratory trial assessed the fate of two $^{15}N$-labelled NUNCs, creatine and hypoxanthine, in a pasture soil. These NUNCs were used because they are precursors to all other NUNCs of focus, as explained above, therefore their degradation in soils is representative of all other NUNCs. It was hypothesised that NUNCs would degrade rapidly in soils and NUNC-N would be present in soil inorganic N and plant N pools within four days, based on the above stated residence times of these compounds in soil. Given that NUNCs were observed to rapidly degrade to soil inorganic N in the laboratory trial, showing their potential to significantly contribute to soil $N_2O$ emissions, a field trial was performed to determine the effect of increasing the proportion of urine N excreted as individual NUNCs on the urine patch EF. It was hypothesised that an increased proportion of urine N excreted as PDs would decrease the urine patch EF due to their stimulatory effect on plant growth, thereby increasing plant N uptake, while excretion of urine N as creatine or creatinine would not alter the urine patch EF.

## 3.2 Materials and Methods

### 3.2.1 $^{15}$N-labelled NUNC laboratory trial

Soil was collected to 10 cm depth from the field trial site (see 3.2.2.1) on February 8, 2017 and stored at 4°C until use. On March 17, 15 mL falcon tubes (140 total; Nest Biotechnology Co. Ltd.) were filled with 10.8 g soil (10 g dry weight equivalent) and seeded with 5 seeds of Base Tetraploid perennial ryegrass (*Lolium perenne*) with AR37 endophyte (PGG Wrightson Seeds). It was estimated that the average soil bulk density (BD; g dry soil $cm^{-3}$) was 1 g $cm^3$, since the 10 g dry weight soil filled approximately 10 $cm^3$ in the falcon tubes. The tubes were placed outside in a complete randomised design for 5 weeks, until plants had reached a height of approximately 10 cm, with water applied as needed to maintain plant growth. On April 30, 72 tubes were selected based on even plant growth and vitality. Plants were cut to a height of 13 cm and tubes were placed into a split-plot design with 4 blocks. From this date onwards, the experimental tubes were incubated inside at 20°C (Figure 3.1).

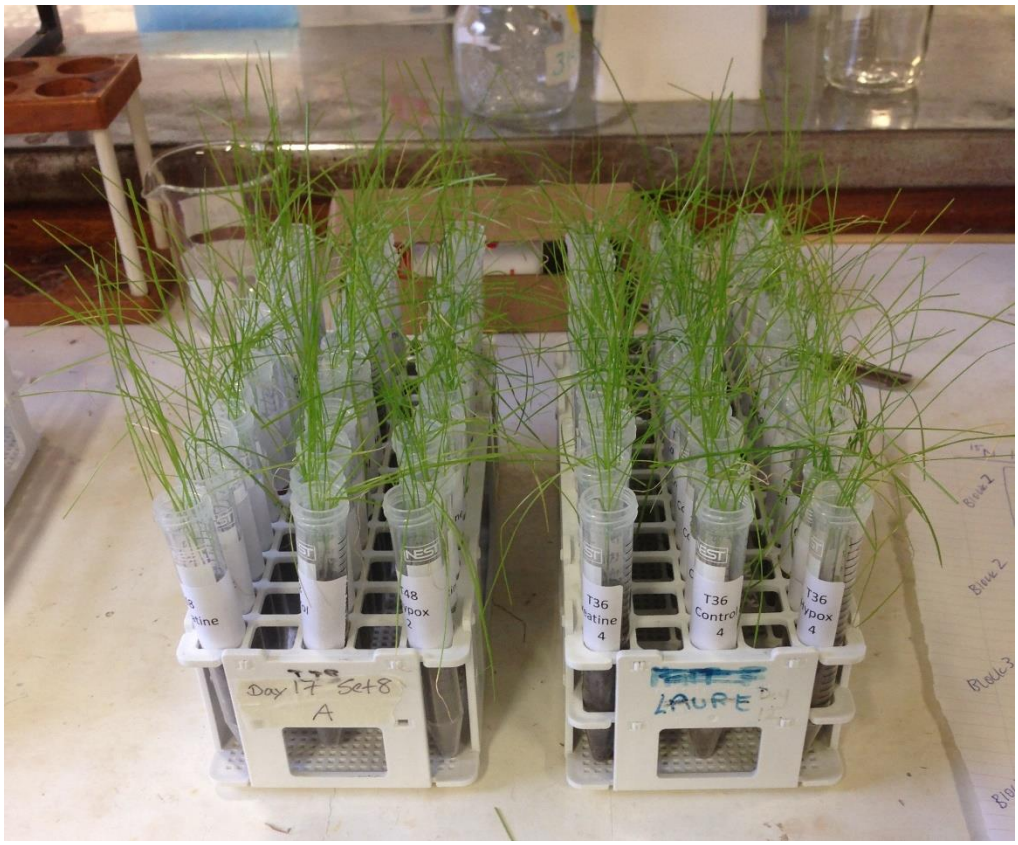



**Figure 3.1 Two sets of the experimental tubes placed into the split-plot design.**

Three treatments (control, creatine, and hypoxanthine) were replicated 4 times, with 6 complete sets for destructive analysis at varying times, giving a total of 72 tubes. Treatments were prepared on

April 30, 2017 and applied at 7:00am on May 1 (Hour 0 of the experiment). Creatine and hypoxanthine occur at quite low concentrations in ruminant urine, therefore, the loading rate of the applied $^{15}N$ treatments was increased in this trial, relative to a typical urine patch loading rate, to ensure the labelled $^{15}N$ compound could be traced. Creatine was applied at 98 µg N $g^{-1}$ soil, approximately 4 times the typical loading rate, and hypoxanthine was applied at 86 µg N $g^{-1}$ soil, approximately 20 times the typical loading rate. The N loading rate for the hypoxanthine treatment was constrained due to the low solubility of the compound, so the results of the creatine and hypoxanthine treatments are reported relative to the control and are not statistically compared to each other.

The two chemicals, both with a $^{15}N$ enrichment of 5 atm %, were dissolved in deionised water: 1.02 mg N $mL^{-1}$ in the creatine treatment and 0.70 mg N $mL^{-1}$ in the hypoxanthine treatment. Each tube received 3 mL of a treatment solution, with the control treatment receiving 3 mL of deionised water at this time (Figure 3.2). All tubes were centrifuged at 500 rpm for 5 min to ensure the treatment was fully absorbed into the soil. After treatment application, soils contained approximately 42% water-filled pore space (WFPS), equivalent to 26% gravimetric water content, and were maintained at this moisture content for the duration of the experiment.

**Figure 3.2 Treatments infiltrating the soil profile immediately after application. Vials were centrifuged for 5 minutes to ensure that no treatment liquid remained pooled on the top of the soil profile.**

Sampling occurred at 12, 24, 36, 48, 72, and 102 hours after treatment application. To measure $N_2O$ emissions, tubes were placed inside a sealed 100 mL vial (93 mL headspace). One 20 mL sample was taken after 1 hour of sealed incubation to determine concentration and enrichment of $N_2O$ in the headspace. Samples were taken with a 30 mL plastic syringe fitted with a 3-way stopcock and transferred to 12 mL glass Exetainers (Labco Ltd.) that had been flushed with helium and evacuated to -1 atm. Fluxes of $N_2O$ were not calculated in this trial because multiple gas samples could not be taken, due the small headspace volume, and the primary focus of the study was to determine if, and when the $^{15}N$ labelled compounds entered the $N_2O$ pool.

At each sampling time, one set of tubes was destructively analysed for plant N content and plant $^{15}N$ enrichment, soil $NH_4^+$ and $NO_3^-$ concentrations, and soil moisture. Gravimetric soil moisture (g water $g^{-1}$ oven dry soil) was determined by placing >3 g soil in a 105°C oven for 24 h, and calculated using Equation 3.1.

**Equation 3.1:** $$\boldsymbol{\theta_g = \frac{M_w - M}{M}}$$

Where:

$\theta_g$ = gravimetric soil water content (g water $g^{-1}$ oven dry soil),

$M_w$= mass of wet soil (g), and

$M$ = mass of oven dry soil (g).

Soil water-filled pore space (WFPS) was calculated using Equation 3.2.

**Equation 3.2:** $$\boldsymbol{WFPS}\ (\%) = \frac{\boldsymbol{BD \times \theta_g}}{\mathbf{1} - (\frac{\boldsymbol{BD}}{\mathbf{2.65}})}$$

Where:

BD = soil bulk density (g oven dry soil $cm^{-3}$), and

$\theta_g$= soil gravimetric water content (g water $g^{-1}$ oven dry soil).

Plant samples were cut at 0.5 cm above the soil surface and dried in a 60°C oven. Dried plant samples were ground using a bead-grinder (TissueLyser II, Qiagen) and analysed for N content, using a GSL elemental analyser (Sercon Ltd.) and for $^{15}N$ enrichment, as explained below.

Soil $NH_4^+$ and $NO_3^-$ concentrations were determined by extracting 4 g (fresh weight) soil with 40 mL 2M KCl. Samples were shaken end-over-end for 1 hr, centrifuged for 10 min at 2000 rpm, and then gravity filtered through Whatman no. 42 filter paper (Blakemore et al. 1981). Samples were frozen at -4°C until analysed. Soil inorganic N samples were analysed using flow injection analysis (FIAstar 5000 Analyser, FOSS Analytical). Soil inorganic N concentrations were determined using Equation 3.3.

**Equation 3.3:** $$\boldsymbol{N = \frac{N_i \times V}{M}}$$

Where:

$N$ = inorganic N content (μg $g^{-1}$ dry soil),

$N_i$ = inorganic N concentration in KCl extract (μg $mL^{-1}$),

$V$ = volume of KCl extract (mL), and

$M$ = weight of dry soil (g).

Only soil samples with $NH_4^+$ concentrations higher than 1.3 μg $NH_4^+$-N $mL^{-1}$ in the KCl extract were further analysed for $^{15}N$ content, due to the detection limits of the instruments. No samples contained sufficient $NO_3^-$-N concentrations for $^{15}N$ analysis. Ammonium samples were prepared for $^{15}N$ enrichment analysis using a routine diffusion procedure (Brooks et al. 1989; Stark & Hart 1996).

To measure $^{15}N$ enrichment in the soil $NH_4^+$ and plant leaf samples, the samples were pre-weighed into tin capsules and combusted at 1000°C in an oxygen atmosphere in a GSL elemental analyser, converting N species into dinitrogen gas, linked to a 20-22 stable isotope ratio mass spectrometer (Sercon Ltd.). The $N_2O$ concentration and $^{15}N$ enrichment of gas samples was measured by preparing the samples using a GSL elemental analyser and TGII trace gas module (Sercon Ltd.) before analysing them on the stable isotope ratio mass spectrometer (see above).

### *3.2.1.1 Laboratory trial data transformations*

Treatment contribution to N pools was calculated with a linear fit between the two points: $x$ = $^{15}$N natural abundance (0.3663 atm %), $y$ = 0 % contribution; and $x$ = treatment $^{15}$N enrichment (5 atm %), $y$ = 100% contribution (Figure 3.3).

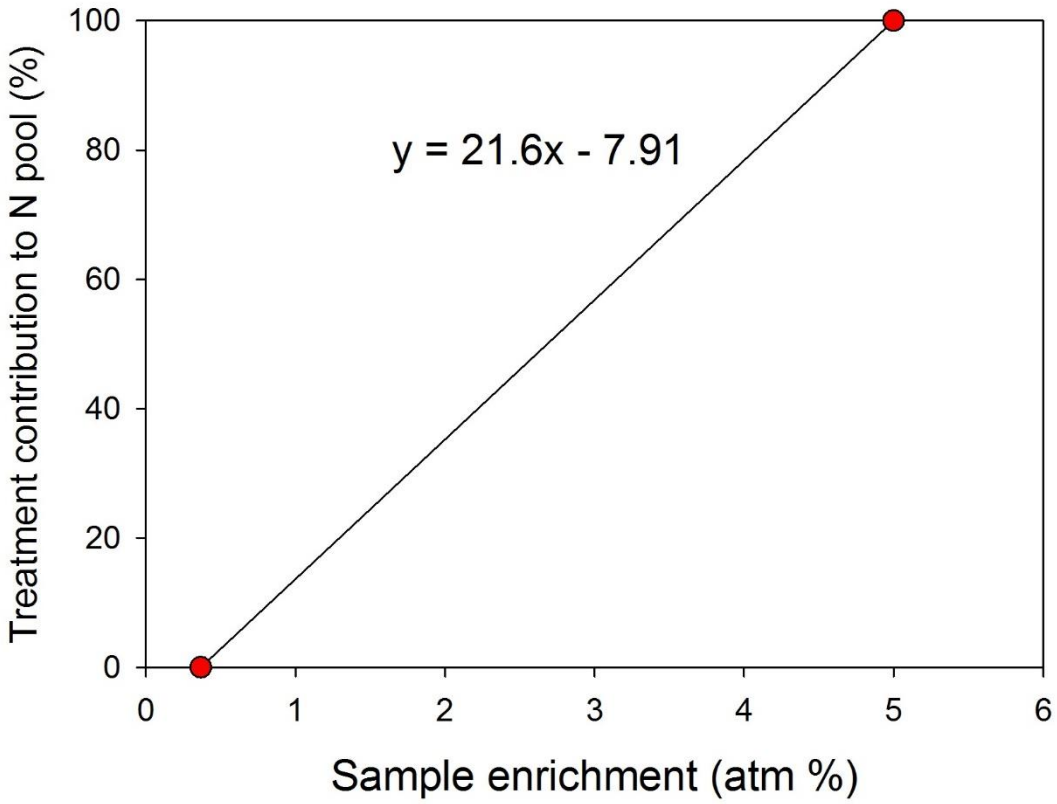



**Figure 3.3 A visualisation of the linear fit calculated between 0 and 100% treatment-N contribution to a given N pool.**

The resulting equation was:

**Equation 3.4:** $$\boldsymbol{y = 21.6x - 7.91}$$

Where:

$y$ = treatment contribution (%), and

$x$ = $^{15}$N enrichment (atm %).

Enrichment data from the soil, plant, and gas pools was entered into Equation 3.4 to determine NUNC-N contribution.

#### *3.2.1.2 Laboratory trial statistical analysis*

Data were analysed using Genstat Version 18, using two-way ANOVA, with treatment and block as the variables. Least significant differences of means (5% level) were used to determine significance using Fisher's unprotected least significant difference test. As stated above, due to the difference in N application rate between treatments, significance was only determined between each treatment and the control, not between treatments. Residual plots generated by the ANOVA analysis in Genstat for each variable were visually analysed to check that the data met the assumptions for the ANOVA analysis. The normality of residuals was checked using the normal plot and half-normal plot. The equal variance of data was determined using the fitted-value plot. Statistical analysis was performed on data sets that met the assumptions of the ANOVA analysis.

### 3.2.2 Urine NUNC composition field trial

#### *3.2.2.1 Field trial site and preparation*

The trial was located at Lincoln University, New Zealand (43°38'37.3"S 172°28'04.1"E) on a PR-WC pasture. The soil type was a Wakanui silt loam (Mottled Immature Pallic Soil (NZ)/Aeric Epiaquept (USDA)) that had a bulk pH in water of 6.1, organic matter content of 52 mg $g^{-1}$, total N content of 2.8 mg $g^{-1}$, and a C:N ratio of 10.6 (0-7.5 cm depth). To avoid carry-over effects of previous urine patch deposition events, the site had not been grazed for one year prior (April 14, 2015), with routine cutting and removal of forage. The final mowing, to a height of 5 cm, occurred 20 days prior to treatment application. Herbage had grown to a height of 10 cm by the day of treatment application (Day 0). Irrigation (25-30 mm) was applied on Day -5 and Day -16 using centre line pivot irrigation.

To monitor $N_2O$ gas fluxes, 28 stainless steel gas chamber bases (40 cm diameter) were installed at the trial site 15 days prior to treatment application. The chamber lids created a ~7.5 L headspace and were insulated with 3 mm polystyrene foam coated with a reflective foil to prevent increases in headspace temperature during the sampling period. The chambers bases were fitted with external annular moats that were filled with water to provide a gas-tight seal when the chamber lid was placed into position. Destructive soil sampling areas (26 cm diameter) immediately adjacent to each chamber base were delineated by wooden marker pegs and managed identically to the pasture within the chamber bases (Figure 3.4).

**Figure 3.4 A gas chamber installed at the field site (right), with a soil sampling area (left) marked by a wooden peg placed in the middle of the 23 cm diameter sampling area. The treatment for this replicate, which was mixed in the laboratory and transported to the field site, is in the white container on the right.**

Sensors were installed into additional treatment areas (Figure 3.5) to monitor soil temperature (107, Campbell Scientific), soil oxygen ($O_2$) (SO-110, Apogee Instruments), and volumetric water content (CS 615, 616, Campbell Scientific). Soil temperature sensors were installed at 10 cm depth in the control and urine treatments (Table 3.1). Soil $O_2$ sensors were installed at 10 cm depth in all treatments and used as a one-point calibration. Each $O_2$ sensor contained a diffusive head, which integrated an area of ~385 $mm^2$ around the sensor. Soil volumetric water content was determined in the control, urine, allantoin, creatinine, and creatine treatments by inserting the probe parallel to the soil surface at 10 cm depth. For comparison with other studies, all soil volumetric water content data were reported as WFPS, calculated using Equation 3.2. Data were monitored using a CR1000 logger equipped with an AM 16/32 multiplexer (Campbell Scientific), with samples taken every 15 min for the duration of the experiment.

**Figure 3.5 Sensor installation at the field trial site. The gas chambers were installed on the mown area on either side of the centre line pivot irrigation pipe, which can be seen running down the middle of the plot area.**

Air temperature, pressure, rainfall, and humidity were monitored using a CR1000 logger, CS215 temperature and relative humidity probe, and a TB3 tipping bucket (0.2 mm). Rainfall data were obtained from a meteorological station from the National Institute of Water and Atmosphere (NIWA) 50 m from the trial site (43°38'51.1"S, 172°28'05.2"E). Logger data from all treatments were averaged to provide overall experimental conditions.

#### *3.2.2.2 Experimental design and treatments*

Seven treatments were arranged in a randomised block design with four replicates. Treatments consisted of a control, a standard synthetic urine treatment, and five NUNC treatments, in which the standard synthetic urine was altered by increasing the concentration of an individual NUNC (Table 3.1). All NUNCs were evaluated in the field trial for their effect on urine patch N dynamics, therefore synthetic urine was used in this experiment to allow for exact manipulation of urine NUNC concentrations.

The standardised synthetic urine was prepared following the methods of Kool et al. (2006a) and contained a baseline level of the five NUNCs of focus (Table 3.1). To make up the five NUNC-amended treatments, the concentration of the particular NUNC concerned was increased to the highest reported level found in the literature (Table 2.1; Selbie et al. 2015), while all other constituents remained at their baseline concentrations. Although this resulted in slightly different N application rate between treatments, differences in N concentrations were accounted for in the data analyses (see 3.2.2.5).

**Table 3.1 The application rate of the five NUNCs used in this study were determined using the highest concentrations found in urine, based upon a review by Selbie et al. (2015). The varying concentrations of NUNCs in the treatments led to the differences in C and N content between treatments.**

| Treatment | Reported concentration (g N $L^{-1}$) | Baseline concentration in synthetic urine (g N $L^{-1}$) | Concentration used in treatment (g N $L^{-1}$) | N application rate (kg N $ha^{-1}$) | C application rate (kg C $ha^{-1}$) |
|---|---|---|---|---|---|
| **Control** | -- | -- | -- | 0 | 0 |
| **Urine** | -- | -- | -- | 767 | 390 |
| **Allantoin** | 0.27-1.5 | 0.75 | 1.5 | 817 | 445 |
| **Creatinine** | 0.08-0.65 | 0.18 | 0.65 | 800 | 430 |
| **Creatine** | 0.12-0.51 | 0.18 | 0.51 | 787 | 411 |
| **Uric acid** | 0.03-0.18 | 0.05 | 0.18 | 775 | 405 |
| **Hypoxanthine** | 0.03-0.09 | 0.05 | 0.09 | 770 | 396 |

The synthetic urine solutions were prepared in the laboratory and immediately transported (500 m) to the field site, whereupon treatments were applied immediately, at 4:00pm on April 13, 2016 (Day 0), at a rate typical of a bovine urination event (Table 2.1; Haynes & Williams 1993; Selbie et al. 2015).

All treatments were applied to both the pasture within the chamber bases (2.21 L) and to the soil sampling areas (0.93 L), with the urine applcation rate equal to 17.5 L $m^{-2}$ for both the gas chambers and soil sampling areas. While applying the treatment to the soil sampling areas, a plastic ring remained firmly pressed onto the ground until the urine had drained into the soil, in order to delineate the treated zone and prevent surface runoff. Treatments were also applied to the plots containing the previously set up automated sensors, using the same application method as for the soil sampling areas.

#### *3.2.2.3 $N_2O$ flux measurement*

Nitrous oxide fluxes were monitored for 35 days after treatment application. Daily $N_2O$ headspace gas samples were taken between 10:00-12:00am in order to obtain $N_2O$ fluxes typical of the daily average (van der Weerden et al. 2013). Headspace gas samples (12 mL) were taken from each chamber at 0, 20, and 40 min after sealing the gas chamber using a 20 mL glass syringe fitted with a 3-way stopcock and immediately transferred to pre-evacuated (-1 atm) 6 mL glass Exetainers (Labco Ltd.). Gas samples were analysed using an automated gas chromatograph system containing an electron capture detector (SRI 8610c GC, SRI Instruments) coupled to an autosampler (Gilson 222XL, Gilson) equipped with a $^{63}$Ni electron capture detector, as previously described by Clough et al. (1996). Peak Simple 4.44 software (SRI Instruments, Torrance, CA) in conjunction with calibrated $N_2O$ standards (0 to 100 $\mu L^{-1}$; BOC, ISO Guide 34 Reference Material Certificate), was used to construct standard curves in order to determine the sample $N_2O$ concentration (PPM). Fluxes of $N_2O$ were calculated using the change in $N_2O$ concentration over time and corrected for using theoretical flux underestimation, as described by Venterea (2010). A predominant linear relationship was observed in the change in headspace $N_2O$ concentration versus time (96% of urine treatment samples had linear $r^2$ values > 0.95), therefore $N_2O$ flux was determined using linear regression for all samples.

#### *3.2.2.4 Soil and pasture measurements*

Soil corers (2.5 cm diameter x 7.5 cm depth) were used to take duplicate soil cores from each soil sampling area on Days 1, 7, 14, 21, 28, and 35. Soil core samplings were spaced to avoid effects due

to previously taken cores. The duplicate cores were mixed, stored at 4°C, and extracted for $NH_4^+$, $NO_3^-$, and DOC content within 48 hours of collection. Soil $NH_4^+$ and $NO_3^-$ were extracted and analysed as explained in 3.2.1.

Soil gravimetric water content was determined according to the methods described in 3.2.1.

Dissolved organic carbon (DOC) was determined by extracting 10 g of fresh soil with 100 mL deionised water. Samples were shaken side-to-side for 30 min at 75 rpm, centrifuged at 3300 rpm for 30 min, and then syringe filtered through 0.45 µm filters (CA+GF, Phenex). All soil extractions were frozen until analysis. DOC samples were analysed for total organic C using a total organic C analyser (TOC-5000A, Shimadzu). Soil DOC concentrations were determined using Equation 3.5.

**Equation 3.5:** $$C = \frac{C_i \times V}{M}$$

Where:

$C$ = dissolved organic C content (µg C $g^{-1}$ dry soil),

$C_i$ = total C concentration in $H_2O$ extract (µg C $mL^{-1}$),

$V$ = volume of $H_2O$ extract (mL), and

$M$ = weight of dry soil (g).

Soil bulk density (g dry soil $cm^{-3}$) within each chamber base was determined at the conclusion of the experiment (Day 36) by inserting stainless steel rings (7.3 cm diameter x 7.5 cm depth) into the soil, drying the soil cores at 105°C until constant weight. Soil diffusivity ($D_p/D_o$) was calculated according to the methods of Moldrup et al. (2013), using soil bulk density and soil water content to determine air-filled porosity, where $D_p$ is the soil-gas diffusion coefficient ($m^3$ soil air $m^{-1}$ soil $s^{-1}$) and $D_o$ is the gas diffusion coefficient in free air ($m^2$ air $s^{-1}$).

Surface pH measurements were taken on Days 1, 2, 3, 5, 7, 10, 14, 21, 28, and 35 from the soil sampling areas using a flat-surface pH electrode (Broadley James Corp.). The pH probe was corrected

for temperature and calibrated using buffer solutions of pH 4 and 7 before each day of measurement.

On Days 16 and 35, hand clippers were used to cut pasture to a height of 5 cm. Soil sampling areas were also cut to 5 cm using a mower to replicate pasture height in the chambers. Pasture samples were dried at 65°C for 48 hours then weighed to determine dry matter yield (DM; kg $ha^{-1}$). Plant samples were then ground and analysed for N content, as described in 3.2.1. Plant N uptake was calculated using Equation 3.6.

**Equation 3.6:** $$\boldsymbol{N_u = [DM(Day\ 16) \times N_c(Day\ 16)] + [DM(Day\ 35) \times N_c(Day\ 35)]}$$

Where:

$N_u$ = N uptake (g N $chamber^{-1}$),

$DM$ = plant dry matter yield (g), and

$N_c$ = plant N content (%).

### *3.2.2.5 Statistical analysis*

Cumulative $N_2O$ emissions were calculated by manually integrating the daily fluxes over time. Emission factors (EFs) were calculated by taking the difference in cumulative $N_2O$-N flux between the chamber concerned and the average cumulative $N_2O$-N flux from the control, then dividing by the total urine N applied in the given treatment (Table 3.1). Soil and plant N data were expressed as percentage of applied N using the same calculation, for total plant N uptake and both the daily and average $NH_4^+$-N and $NO_3^-$-N concentrations.

ANOVAs were performed using Genstat Version 18 with treatment and block as factors and the given measurements as the variable. Only variables expressed as percentage of N applied (EF, soil N data, and plant N uptake) were analysed using ANOVAs in order to account for the differences in N application rates. Residual plots generated by the ANOVA analysis in Genstat for each variable were visually analysed to check that the data met the assumptions for the ANOVA analysis. The normality of residuals was checked using the normal plot and half-normal plot. The equal variance of data was

determined using the fitted-value plot. Statistical analysis was performed on data sets that met the assumptions of the ANOVA analysis. ANOVAs were performed without the control for the soil N and plant N data, due to skew added to the residual distribution from the control data. Least significant differences of means (5% level) were used to determine significance using Fisher's unprotected least significant difference test.

## 3.3 Results

### 3.3.1 $^{15}$N-labelled NUNC laboratory trial

Differences in $N_2O$ concentration in the headspace on all sampling days were not significant between the control and creatine or hypoxanthine treatments (Figure 3.6). The $^{15}N$ enrichment of $N_2O$ in the headspace increased in the hypoxanthine treatment by 102 h, compared to the control ($P<0.05$), with the applied treatment-N accounting for 3.2% of headspace $N_2O$-N.

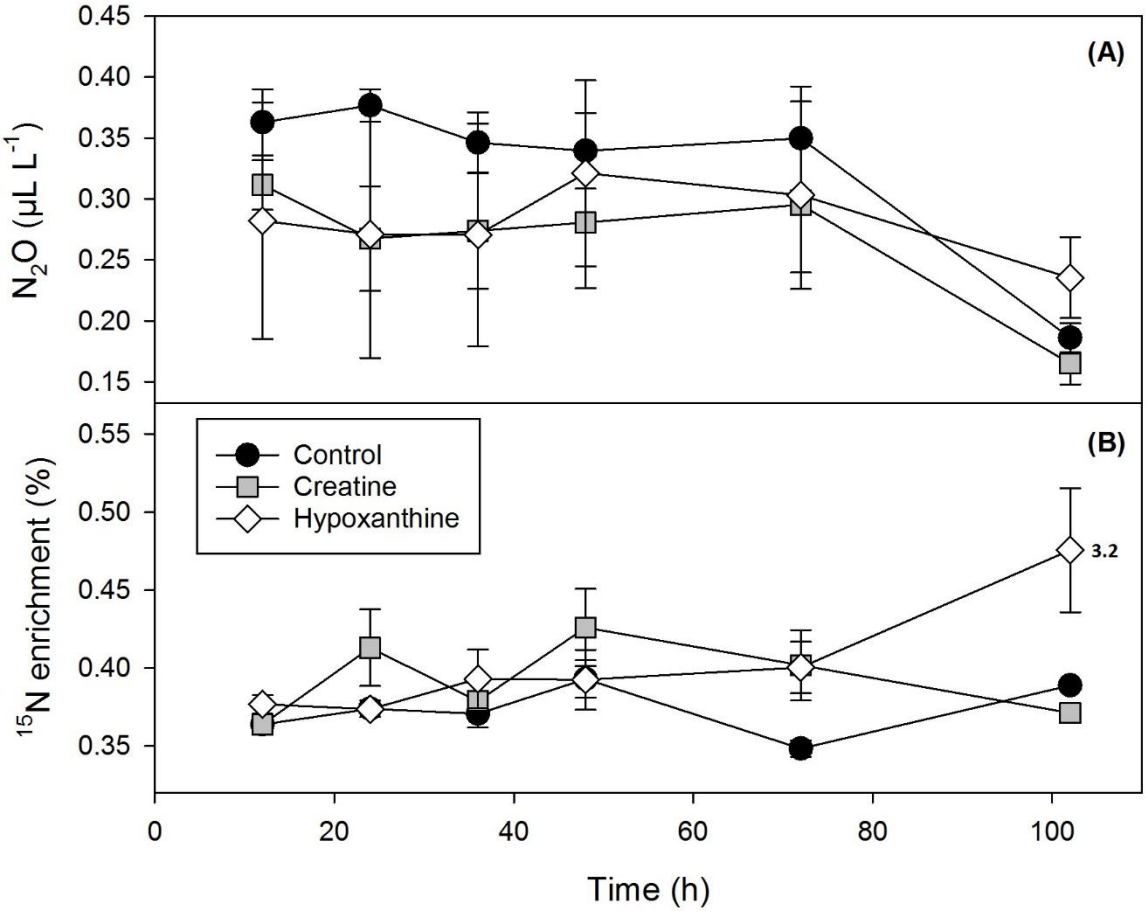



**Figure 3.6 Nitrous oxide concentrations (µL N $L^{-1}$) in the headspace after incubating vials in a closed headspace for 60 min (A) and the $^{15}N$ enrichment (atm %) of the $N_2O$ in the headspace at this time (B). Data points are means (n=4) with error bars representing the standard error of the mean (SEM). The calculated % contribution of treatment N to soil $N_2O$-N is included next to the data points in (B), as described in 3.2.1.1. N contribution is only included for data points where treatment $N_2O$-N concentrations were significantly higher than the control.**

Soil $NH_4^+$ and $NO_3^-$ concentrations increased within 12 hours in the hypoxanthine treatment and within 48 hours in the creatine treatment, when compared to the control ($P<0.05$) and remained higher for the remainder of the experiment (Figure 3.7A,B). Soil $NH_4^+$ concentrations peaked 24 hours after applying the hypoxanthine treatment, with hypoxanthine-N calculated to have contributed to 73% of the $NH_4^+$-N pool (Figure 3.7A). The hypoxanthine contribution to the soil $NH_4^+$ pool peaked at 36 hours, with 88% of soil $NH_4^+$-N calculated to be derived from hypoxanthine. At 72 hours, soil $NH_4^+$ concentration in the creatine treatment reached its peak, with a calculated 94% creatine-N contribution to the $NH_4^+$-N pool (Figure 3.7A).

Soil $NO_3^-$ concentrations peaked at 72 hours in the hypoxanthine treatment, and at 102 hours in the creatine treatment (Figure 3.7B), however, treatment-N contributions to $NO_3^-$ concentrations could not be determined because the soil samples did not contain sufficient $NO_3^-$-N concentrations for $^{15}N$ analysis, as stated in 3.2.1.1.

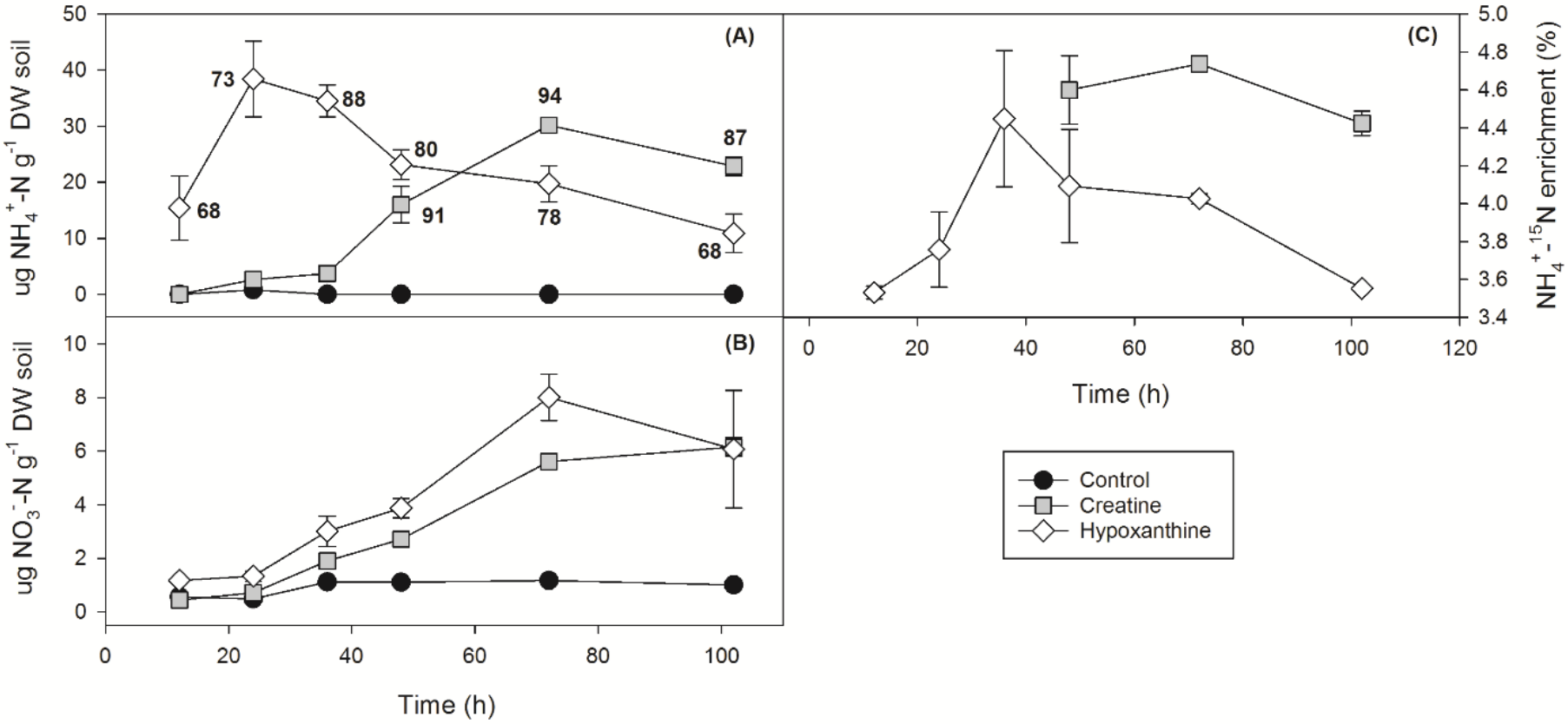



**Figure 3.7 Soil $NH_4^+$-N and $NO_3^-$-N concentrations expressed per gram soil (A and B). Soil $^{15}NH_4^+$ enrichment (C) is given for samples that contained sufficient $NH_4^+$-N concentrations, as explained in 3.2.1. Data points are means (A and B: n=4; C: n=1-4) with error bars (SEM). The calculated % contribution of treatment N to soil $NH_4^+$-N is included next to the data points in (A), as described in 3.2.1.1. N contribution is only included for data points where treatment $NH_4^+$-N concentrations were significantly higher than the control.**

Plant N content increased in the hypoxanthine and creatine treatments, compared to the control, within 12 and 36 hours of treatment application, respectively ($P<0.05$; Figure 3.8A). Plant-N in both treatments remained higher than in the control for the remainder of the experiment ($P<0.05$).

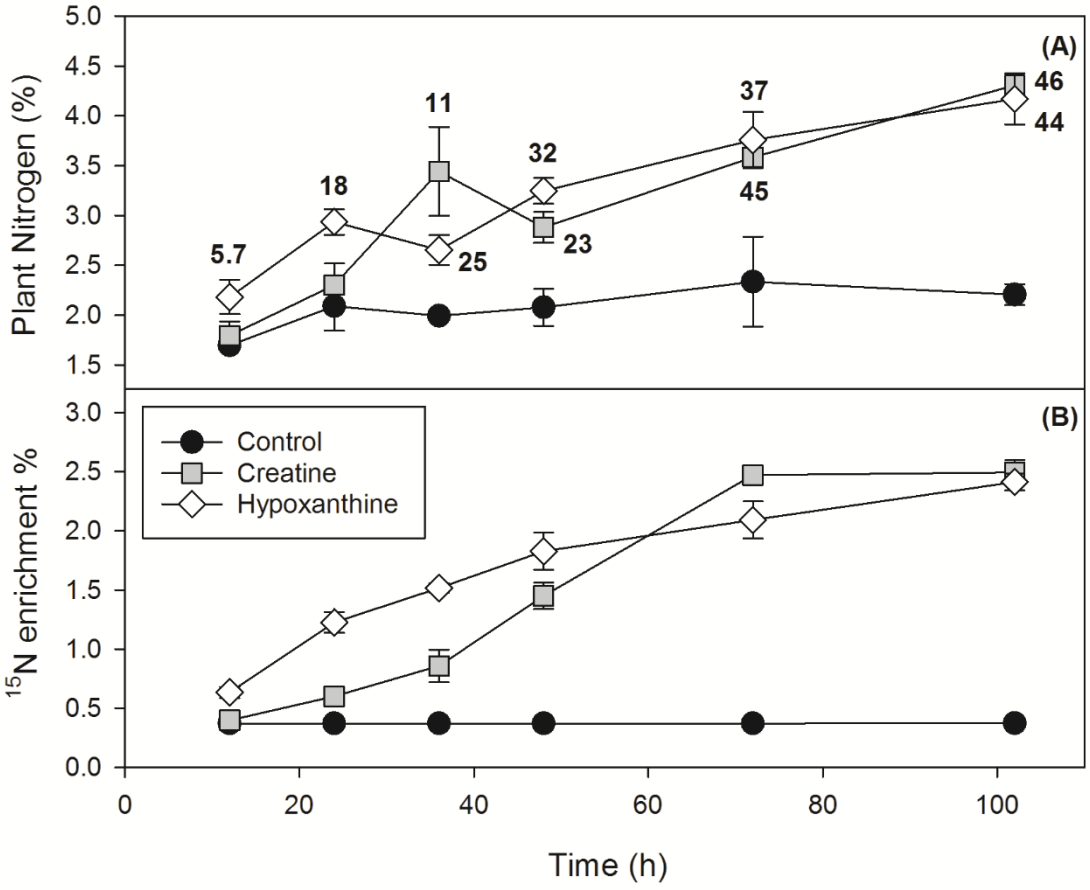



**Figure 3.8 Plant N content, expressed as percentage dry matter (A), and the $^{15}$N enrichment of plant N (B) at each sampling time. Data points are means (n=4) with error bars (SEM). The calculated % contribution of treatment-N to plant-N is included next to the data points in (A), as described in 3.2.1.1. N contribution is only included for data points where plant-N concentrations were significantly higher than the control.**

Plant N enrichment increased ($P<0.05$) in the hypoxanthine treatment within 12 hours and within 24 hours in the creatine treatment after treatment application, when compared to the control (Figure 3.8B). The calculated contribution of treatment-N to plant-N content peaked at 102 hours, with hypoxanthine and creatine-N contributing to 44% and 46% of plant-N, respectively (Figure 3.8A).

### 3.3.2 Urine NUNC composition field trial

#### *3.3.2.1 Soil and Climate Properties*

During the 35 day trial, air temperature averaged 12.8°C (1.4-23.2) and soil temperature averaged 13.4°C (11.3-15.4) (range in brackets; Figure 3.9A). Rainfall totalled 16.5 mm and occurred on 5 occasions (Days 2, 6, 12, 16, and 22). Plots were irrigated with 19.5 mm on Day 23. During the experiment, WFPS ranged from 44.7-60.1%, with an average of 52.5% (Figure 3.9C). Soil bulk density averaged 1.20 g $cm^{-3}$ and did not vary significantly between treatments. Soil $D_p/D_o$ ranged from 0.009-0.050.

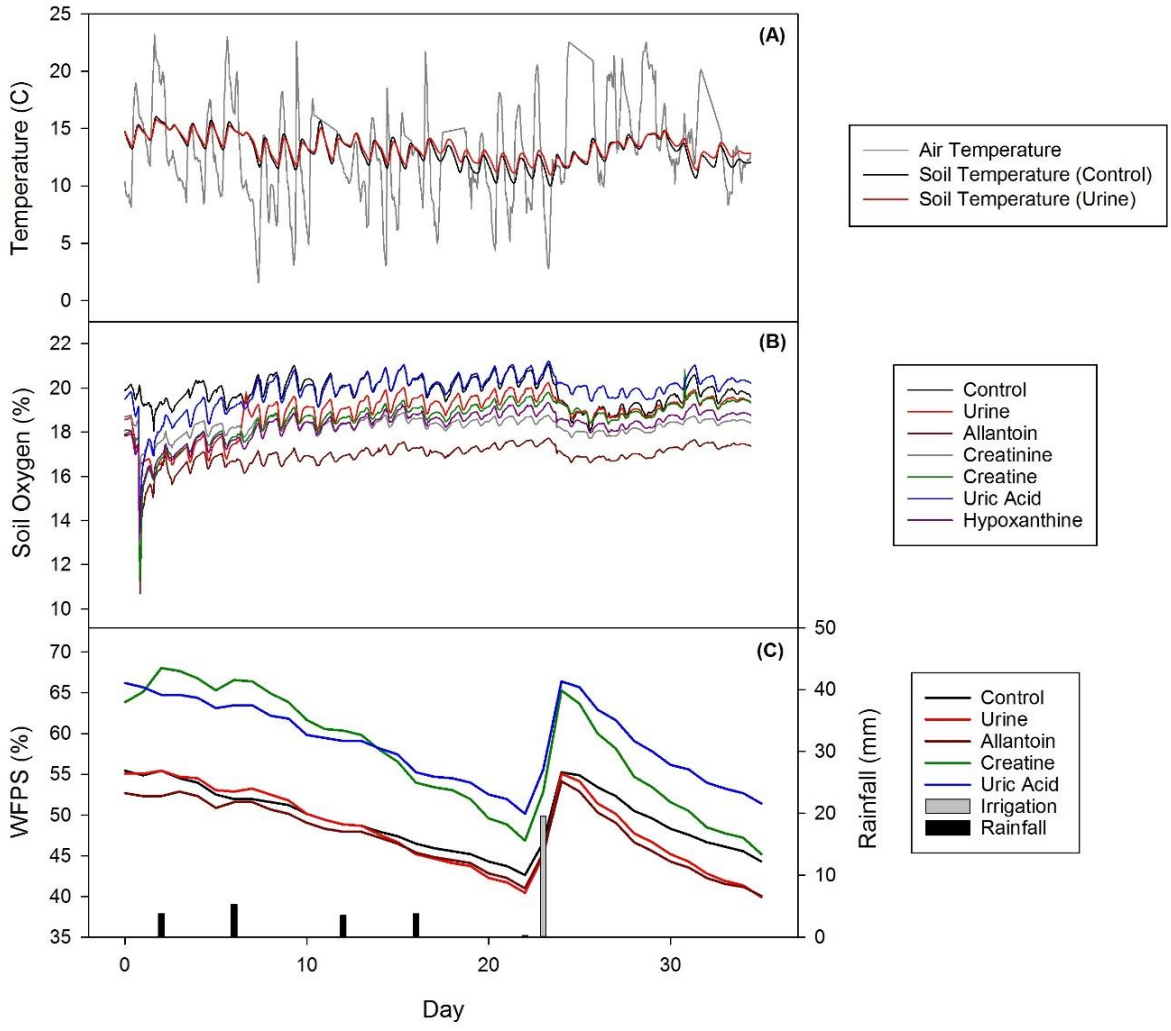



**Figure 3.9 Logger data for temperature (A), soil oxygen (B), and soil water-filled pore space (WFPS; C) for Days 0 to 35. WFPS was calculated using Equation 3.2, using volumetric water content as the numerator rather than gravimetric water content and bulk density. All logger data is reported at 1 hour intervals. Irrigation and rainfall are given as daily totals.**

#### *3.3.2.2 Nitrous oxide fluxes*

Daily $N_2O$ flux rates peaked on Day 5 and overall ranged from 0-153 g $N_2O$-N $ha^{-1}$ $d^{-1}$, varying with treatment over time (Figure 3.10A). The $N_2O$ EF for the uric acid treatment (0.12%) was higher than the EF for the creatine (0.09%) and creatinine (0.08%) treatments ($P<0.05$), however, no treatment EF was significantly different than the urine EF (0.09%) (Figure 3.10B).

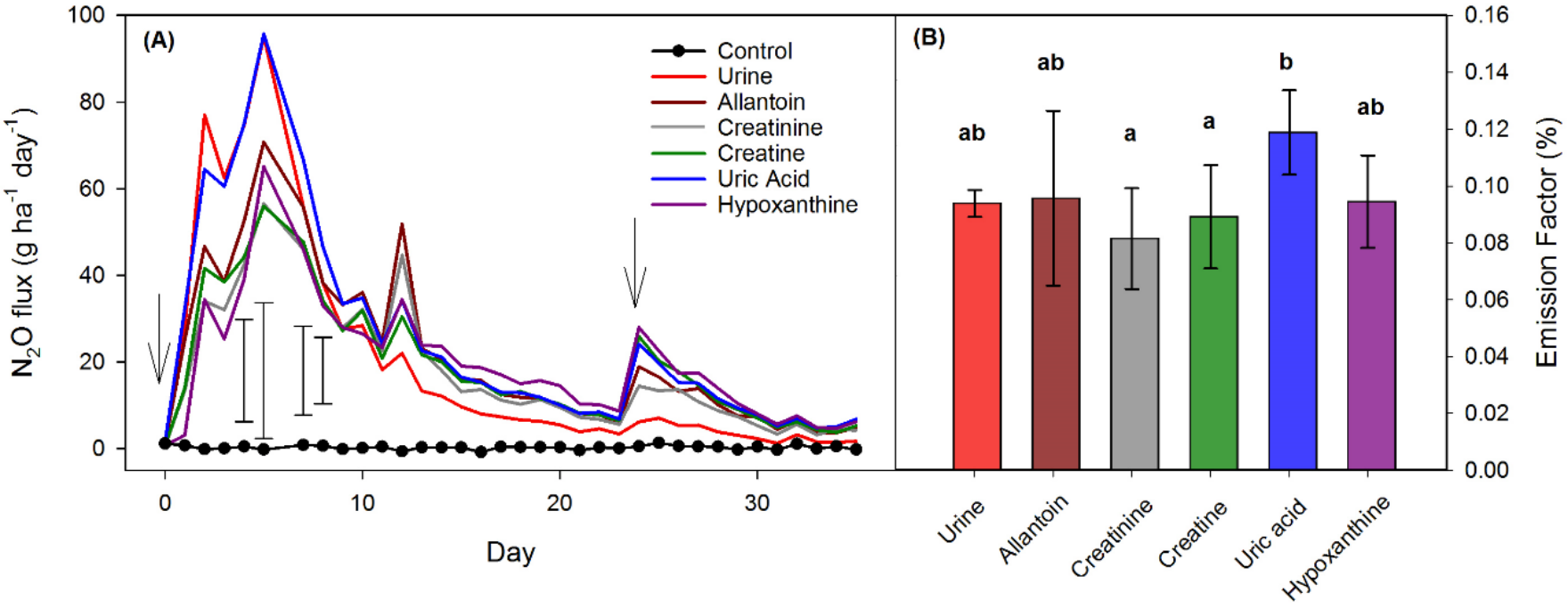



**Figure 3.10 (A) Mean daily $N_2O$ flux from the pasture soil over the 35 day field trial (n=4). LSDs (5%) for Days 4, 5, 7, and 8 are included as examples of daily variation. LSDs were used rather than SEM to reduce clutter. LSDs were calculated excluding the control. Symbols are included on the control treatment to indicate sampling days. Arrows indicate timing of treatment application (Day 0) and irrigation application (Day 23). (B) The emission factor for each treatment (n=4), as a percent of total applied N, with error bars (SEM).**

During the initial $N_2O$ peak period (Days 0-14), the creatinine, creatine, and hypoxanthine treatments had lower cumulative $N_2O$ emissions, as a percentage of N applied, when compared to the uric acid treatment ($P<0.05$). The urine treatment did not differ from any of these treatments during this time. Over Days 14-35, the urine treatment had lower $N_2O$ emissions, as a percentage of applied N, than the hypoxanthine treatment ($P<0.05$), but was not different from any other NUNC treatment.

#### *3.3.2.3 Responses of soil and plant variables*

Overall, soil $NH_4^+$ and $NO_3^-$ concentrations peaked on Day 1 and on Day 21, respectively (Figure 3.11A,B). The average soil $NH_4^+$-N concentration, expressed as a percentage of applied N, was higher in the urine treatment compared to the allantoin and uric acid treatments ($P<0.05$; Table 3.2). However, the daily NUNC treatment soil $NH_4^+$-N concentrations, as a percentage of N applied, only differed from the urine treatment on Day 7, when the allantoin treatment had a significantly lower $NH_4^+$-N concentration (Figure 3.11C).

The urine treatment contained higher $NO_3^-$-N concentrations compared to the hypoxanthine treatment on Day 7, and compared to the allantoin treatment on Days 21 and 35 ($P<0.05$; Figure 3.11D). The allantoin treatment was the only NUNC treatment that had lower average soil $NO_3^-$-N concentration, as a percentage of applied N, compared to the urine treatment ($P<0.05$; Table 3.2).

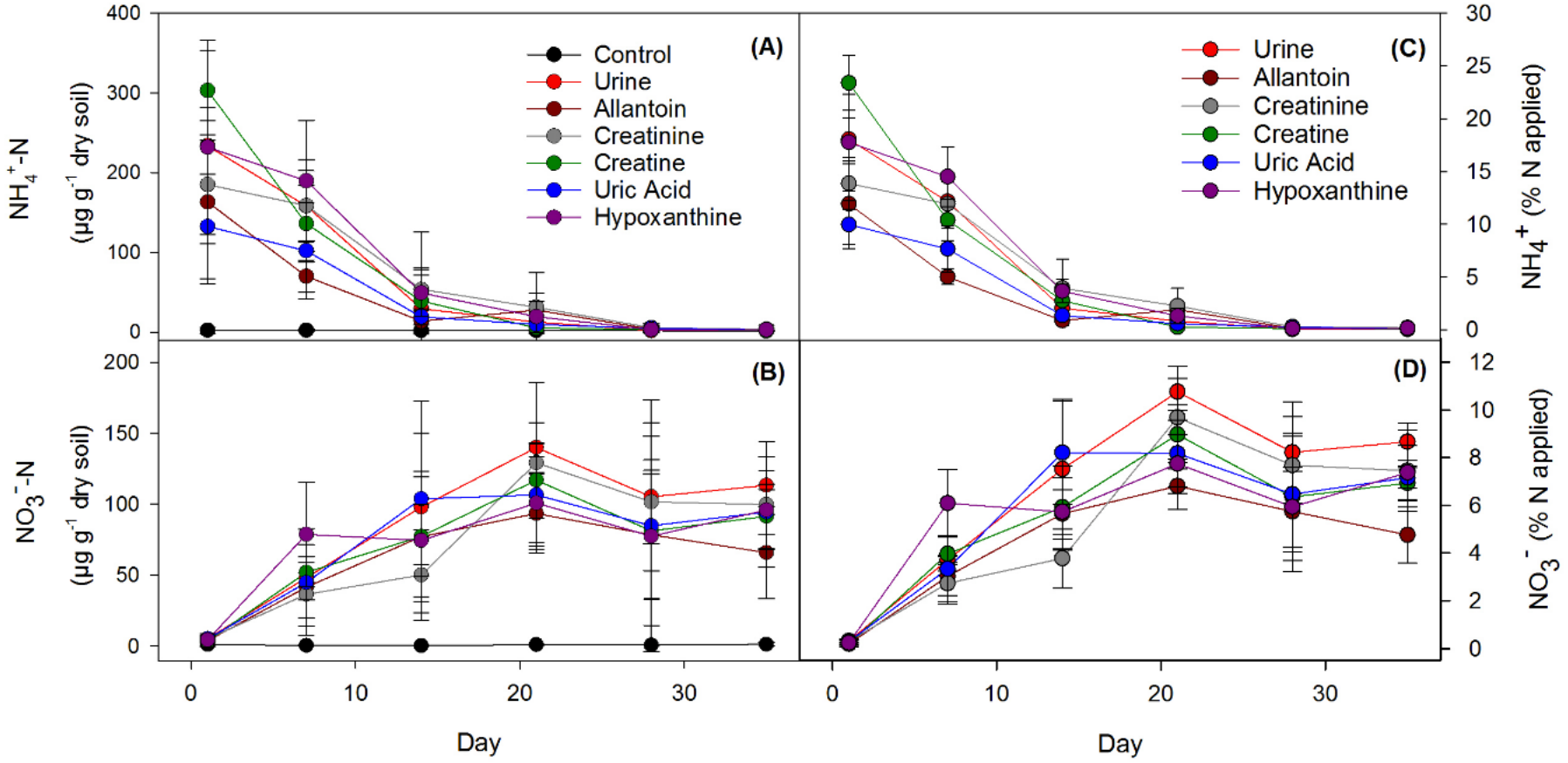



**Figure 3.11 Changes in soil $NH_4^+$-N and $NO_3^-$-N, expressed per gram soil (A and B) or as a % of the N applied in each treatment (C and D). The calculations for % N are explained in *2.2.4*. Data points are means (n=4) with error bars (SEM).**

**Table 3.2 Mean values (n=4) over the 35 day trial of soil inorganic N, plant dry matter (DM), and plant N uptake. Soil inorganic N and plant N uptake are presented as percentage of applied N, as explained in 3.2.2.5. No statistical analysis was performed on total plant DM because these values do not account for differences in N applied between treatments.**

| Treatments | $NH_4^+$-N (% applied N) | $NO_3^-$-N (% applied N) | Total Plant DM (kg $ha^{-1}$) | Plant N uptake (% applied N) |
|---|---|---|---|---|
| **Control** | - | - | 1780 | - |
| **Urine** | 5.5 $^{b}$ | 6.5 $^{b}$ | 2750 | 7.9 $^{a}$ |
| **Allantoin** | 3.3 $^{a}$ | 4.4 $^{a}$ | 2660 | 6.5 $^{a}$ |
| **Creatinine** | 5.4 $^{ab}$ | 5.3 $^{ab}$ | 2500 | 6.5 $^{a}$ |
| **Creatine** | 6.2 $^{b}$ | 5.4 $^{ab}$ | 2710 | 7.3 $^{a}$ |
| **Uric acid** | 3.3 $^{a}$ | 5.6 $^{ab}$ | 2850 | 7.3 $^{a}$ |
| **Hypoxanthine** | 6.2 $^{b}$ | 5.5 $^{ab}$ | 2680 | 7.5 $^{a}$ |
| **LSD (5%)** | 2.2 | 2.1 | - | 3.0 |

Overall, urine treatments increased plant dry matter (DM) compared to the control (Table 3.2). Plant N concentration ranged from 3.7-4.4% on Day 16 and 4.7-5.1% on Day 35 in the urine and NUNC treatments, with no significant difference between treatments on either day. Total plant N uptake, as a percent of applied N, did not differ between treatments (Table 3.2).

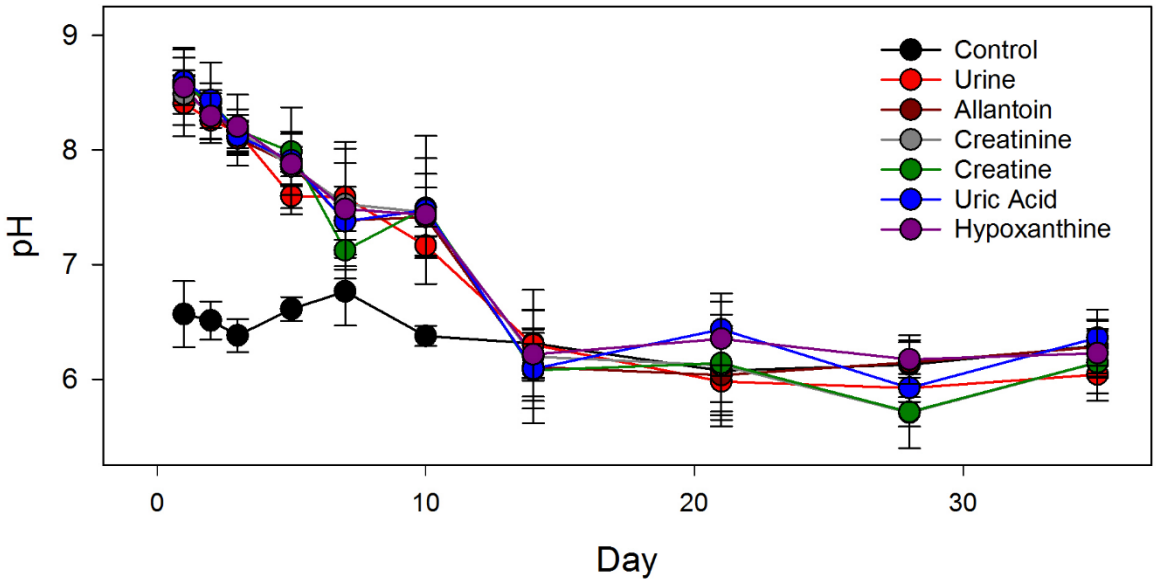



**Figure 3.12 Soil surface pH during the 35 day field experiment. Data points are means (n=4) with error bars (SEM).**

From Days 1 to 14, soil surface pH was higher in the urine and NUNC treatments than the control, peaking on Day 1, before decreasing to a pH similar to the control from Day 14 to 35 (Figure 3.12). Surface pH averaged 6.69 in the control and 7.22 for all urine treatments over the 35 day period.

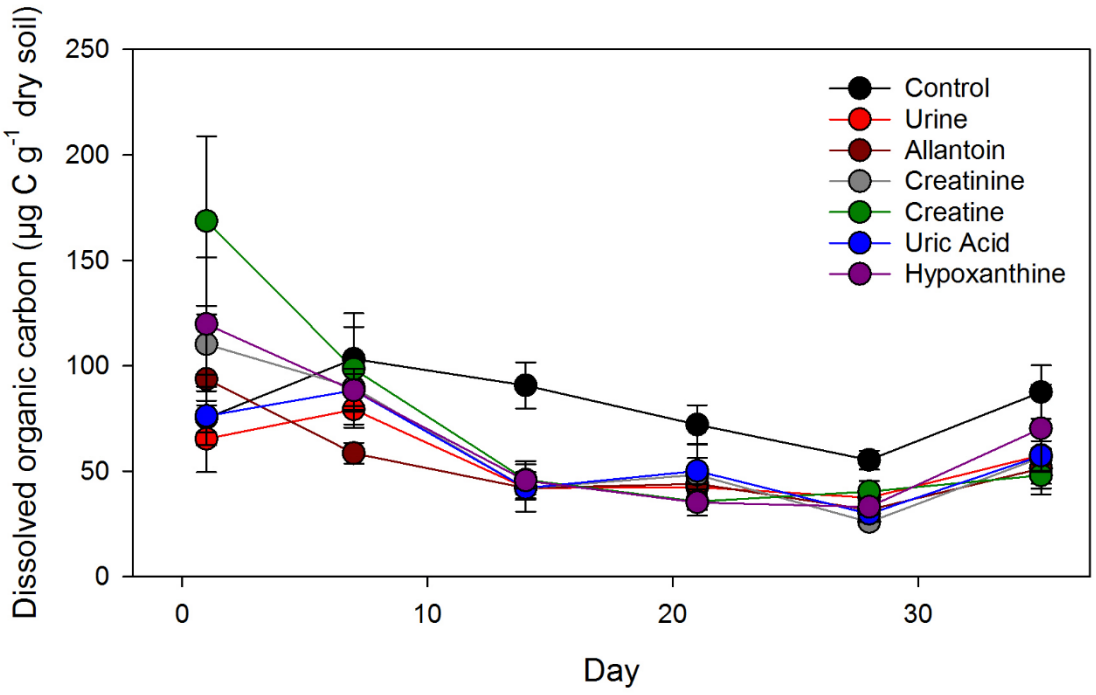



**Figure 3.13 Soil dissolved organic carbon concentrations during the 35 day field experiment. Data points are means (n=4) with error bars (SEM).**

The control treatment contained the highest concentration of DOC when averaged over the duration of the experiment, due to higher concentrations from Days 14 to 35 (Figure 3.13). However, overall average DOC concentrations between the urine and NUNC treatments were not significantly different.

## 3.4 Discussion

### 3.4.1 Degradation and fate of NUNCs under laboratory conditions

As evidenced by the soil inorganic N dynamics and the $^{15}N$ data, it is clear that hypoxanthine and creatine degraded to form $NH_4^+$ and $NO_3^-$, or were assimilated as plant N, within 4 days of application onto soils. Hypoxanthine-N contributed to the soil inorganic N and plant N pools faster than creatine, however, hypoxanthine-N only contributed to 68-88% of soil $NH_4^+$-N, indicating that other $NH_4^+$ producing pathways, such as mineralisation, may have been stimulated by treatment application.

From 24 to 72 h, soil $NH_4^+$ in the hypoxanthine treatment decreased by 19 µg N $g^{-1}$ soil, while soil $NO_3^-$ only increased by 6.7 µg N $g^{-1}$ soil. This discrepancy suggests that a proportion of $NH_4^+$ was removed via plant uptake, which was shown in the plant N data, or removed via immobilisation or ammonia volatilisation (Sherlock 1984). Since the latter two were not measured in this trial, their influence can only be speculated. The decrease in $^{15}N$ enrichment of the $NH_4^+$ pool over time could

be due to $^{15}NH_4^+$ removal via these pathways, combined with $NH_4^+$ influx from mineralisation stimulated by treatment application.

It has been shown that, at 50% WFPS, autotrophic nitrification contributes to a greater proportion of $N_2O$ emissions than denitrification (Bateman & Baggs 2005). Although the measured increase in soil $NO_3^-$ during this trial shows that nitrification occurred, the rate of nitrification may not have been sufficient for significant $N_2O$ emissions, since $NH_4^+$ was being removed through other pathways, as described above. However, it is clear that hypoxanthine contributed to $N_2O$ emission at 102 h, shown by the significant increase in $^{15}N$-labelled $N_2O$, which indicates that urine N excreted as PDs could be degraded to produce $N_2O$ within 102 hours of their application onto these soils.

Under urine patch conditions, 80-90% of urea-N is hydrolysed to $NH_4^+$-N within 48 hours (Williams & Haynes 1994). Using the measured soil $NH_4^+$-N concentrations and calculated treatment contribution to soil $NH_4^+$-N, it can be inferred that within 48 hours, 35% of hypoxanthine-N and 22% of creatine-N was available as $NH_4^+$-N. Therefore, the results of the laboratory trial indicate that creatine and hypoxanthine are potentially not as readily available as N substrate for soil $N_2O$ emissions as urea-N within 48 hours of urine deposition, thus indicating that an increased proportion of urine N excreted as NUNCs may result in a delayed peak in $N_2O$ emissions after urine application compared to urea. However, the results of the secondary field trial, discussed below, did not show this effect.

Since these compounds were applied to soils at concentrations higher than typical urine patches, their total contribution to N pools is likely inflated compared to typical urine patch conditions. However, the goal of this trial was to determine the fate of these compounds in the urine patch, and to compare their residence time and lability in soil compared to urea. Both hypoxanthine and creatine degraded within their previously reported residence times (Dubos & Miller 1937; Wang et al. 2007).

### 3.4.2 Effects of NUNC treatments on the $N_2O$ EF under field conditions

Contrary to the hypothesis that an increased proportion of urine N excreted as PDs (allantoin, uric acid, or (hypo)xanthine) would reduce the urine patch EF due to their stimulation of plant N uptake, there was no difference in plant N uptake or $N_2O$ EF between the urine and NUNC treatments (Figure 3.10B, Table 3.2). The average percent of applied-N present as $NH_4^+$-N or $NO_3^-$-N in the allantoin treatment was significantly lower than the urine treatment, however, these differences did not result in a reduction in the $N_2O$ EF. Additionally, no NUNC treatment exhibited signs of nitrification inhibition, typically shown by a delay in the nitrification of $NH_4^+$, along with lower $N_2O$ emissions (Di & Cameron 2002a, 2003; Di & Cameron 2016).

### *3.4.2.1 Factors influencing the field trial $N_2O$ EFs*

The EF values obtained were lower than New Zealand's country-specific EF value of 1%, but are within the previously reported range of EF values for urine patches on pasture soils (Selbie et al. 2015; Owens et al. 2016) and those for free-draining soils (de Klein et al. 2014b). Although $N_2O$ fluxes had returned to background levels by the end of the study, $NO_3^-$ concentrations in soil were still elevated, indicating that there was N substrate available to potentially produce $N_2O$ emissions after the measurement period ended. However, there was no significant difference in $NO_3^-$ concentrations between NUNC treatments on Day 35, indicating that $N_2O$ emitted after the measurement period would be similar between all NUNC treatments.

The low EF values obtained could also be attributed to the use of synthetic urine, which may produce lower EFs than real urine, however, such differences are not always significant (de Klein et al. 2003). Although synthetic urine cannot replicate exactly the real urine conditions, it can be used to compare treatment effects (Anger et al. 2003; Clough et al. 2003; van Groenigen et al. 2005a; de Klein et al. 2014b), and it was necessary to deploy this method to enable the exact manipulation of the urine constituents (de Klein et al. 2003; Kool et al. 2006b). The only other way to do this would involve manipulation of ruminant diet, a resource intensive process with no guarantee of the resulting urinary constituent composition being what was needed.

Low $N_2O$ emissions could also be due to the relatively dry soil conditions during the experiment period. Emissions of $N_2O$ reportedly increase over a soil WFPS range of 55-75% (van Groenigen et al. 2005b), and the average WFPS value ranged from 45-60% in this experiment. Diffusivity ($D_p/D_o$) has been shown to predict the onset of $N_2O$ emissions well, with a value of 0.005 to 0.006 reported to result in elevated emissions (Balaine et al. 2013; Balaine et al. 2016; Owens et al. 2016). Diffusivity values for this experiment ranged from 0.009-0.050, indicating the soil was generally aerobic in the 0-7.5 cm depth. However, following irrigation, average $D_p/D_o$ decreased from 0.045 on Day 22 to 0.015 on Day 24, dropping below the reported threshold of <0.02, where anaerobic soil conditions occur (Stepniewski 1981) and thus where nitrifier-denitrification or denitrification may commence. This may explain the increase in the $N_2O$ flux at Day 24 (Figure 3.10), with the return of $D_p/D_o$ to a value of 0.02 by Day 26 explaining the resulting decline in $N_2O$ emissions. Therefore, the low rainfall, leading to high diffusivity of oxygen, could also be an explanation for the low cumulative $N_2O$ emissions measured.

### *3.4.2.2 Plant N uptake and N loss pathways affecting inorganic substrate supply*

The lack of any significant difference in plant N uptake between the urine treatment and NUNC treatments refutes the hypothesis that allantoin, and its precursor PDs (hypo)xanthine and uric acid,

would stimulate plant growth and therefore increase plant N uptake. It is known that urine deposition stimulates plant growth by adding high concentrations of plant-available N to the soil, therefore the stimulating effects of allantoin could have been masked by the overall increased plant growth and plant N uptake found in urine patches (Haynes & Williams 1993; Buckthought 2014; Buckthought et al. 2016). It is possible that higher concentrations of allantoin could produce a significant effect, however, it is not reasonable to test concentrations above known urinary excretion rates.

Other urine patch N loss pathways, such as ammonia ($NH_3$) volatilisation or dinitrogen ($N_2$) gas emissions, could also have been affected by the varying NUNC treatments. However, the lack of any difference in surface soil pH demonstrates that $NH_3$ volatilisation would also have been comparable between NUNC treatments (Ferguson et al. 1984; Venterea et al. 2015), while the $D_p/D_o$ values indicate soil conditions were too oxic for denitrification, hence the potential for treatment induced losses of $N_2$ via biological denitrification were also unlikely to be significant (Balaine et al. 2016).

#### *3.4.2.3 Effect of variations in applied C on DOC and $N_2O$ emissions*

Rates of C applied also varied as a consequence of treatment, ranging from 390-445 kg $ha^{-1}$, and could potentially have caused the slight, yet not significant, variation in $N_2O$ emissions after the Day 23 irrigation event. Heterotrophic denitrification is promoted under anaerobic conditions and with $NO_3^-$ present. These conditions were both observed after the irrigation event and DOC provided an energy source for the heterotrophic denitrifying bacteria (Burford & Bremner 1975; McCarty & Bremner 1993; Siemens et al. 2003). However, there were no significant differences in DOC concentrations between treatments, suggesting that treatment effects on C rate applied did not translate into significant differences in DOC availability (Table 3.2) and/or $N_2O$ flux. Had soil conditions been anaerobic for longer periods in the study, a treatment difference may have been observed in DOC availability and $N_2O$ flux. It may therefore be warranted to repeat this study under wetter soil conditions.

## 3.5 Conclusions

Increasing the proportion of urine N excreted as NUNCs did not alter the urine patch EF, since these compounds were rapidly degraded in soils and/or lack the ability to inhibit nitrification. The hypothesised plant stimulatory effects of allantoin and its precursor PDs, (hypo)xanthine and uric acid, did not result in increased plant N uptake in the field trial, most likely because plant growth is already stimulated by high soil N concentrations after urine application. Therefore, urine N excreted

as NUNCs contributed to the urine patch EF in a similar manner to N excreted as urea, as demonstrated by the rapid formation of inorganic N in the soil. Hence, it was concluded that varying concentrations of these compounds in ruminant urine does not lead to significant variations in urine patch $N_2O$ emissions.

# Chapter 4
# Potential inhibition of urine patch nitrous oxide emissions by *Plantago lanceolata* and its metabolite aucubin

*A manuscript from this study has been published in the New Zealand Journal of Agricultural Research: Gardiner CA, Clough TJ, Cameron KC, Di HJ, Edwards GR, de Klein CAM 2017. Potential inhibition of urine patch nitrous oxide emissions by Plantago lanceolata and its metabolite aucubin. New Zealand Journal of Agricultural Research: doi: 10.1080/00288233.2017.1411953. This is referred to in later chapters as Gardiner et al. (2017).*

## 4.1 Introduction

Ruminant urine patches are $N_2O$ emission hot-spots and the $N_2O$-producing processes in urine-affected soil are well recognised (Selbie et al. 2015). Previous research has found that compounds which inhibit nitrification in urine-affected soils, a key step in soil $N_2O$ production, can significantly reduce $N_2O$ emissions (Di & Cameron 2002a, 2003). However, these chemicals, such as dicyandiamide (DCD), are currently not available for commercial use in New Zealand (Di & Cameron 2016).

Some plant species contain plant secondary metabolites (PSMs) that can reduce nitrification rates in soils (Subbarao et al. 2006; Subbarao et al. 2007; Skiba et al. 2011; Byrnes et al. 2017). These inhibitory PSMs typically enter soils through root exudation (Subbarao et al. 2007). However, it has also been shown that grazing ruminants excrete PSMs from the pasture plants they consume (Keir et al. 2001). Thus, in grazed pasture conditions, PSMs could potentially enter soil in large quantities through livestock consumption of PSM-containing plants and subsequent excretion of these PSMs in urine or faeces. If inhibitory PSMs were excreted in urine, they would be applied within the urine patch and could reduce nitrification and subsequent $N_2O$ emissions (Gardiner et al. 2016). Hence, pasture forages should be evaluated and selected for PSMs that inhibit or reduce nitrification in pasture soils, either through root exudation of active inhibitory PSMs into pasture soil or through ruminant ingestion of active inhibitory PSMs and their subsequent deposition in urine onto the pasture soil. The effect of these PSMs on urine patch $N_2O$ emissions requires quantification.

One pasture forage identified for its potential to contain inhibitory PSMs is plantain (*Plantago lanceolata*) which contains: acteoside, aucubin, and catalpol (Stewart 1996; Tamura & Nishibe 2002; Gardiner et al. 2016). Recent studies have shown that $N_2O$ emissions after ruminant urine deposition onto pasture swards containing plantain are significantly reduced compared to pasture swards of

PR-WC (Di et al. 2016; Luo et al. 2018). However, these studies did not determine the mechanism of this reduction.

To date, one study has demonstrated that applying plantain leaf extract (PLE) can significantly inhibit nitrification, as shown by a significant reduction in soil nitrate ($NO_3^-$) concentrations in soil, for 56 days after application, likely due to the activity of aucubin or its aglycone: aucubigenin (Dietz et al. 2013). Aucubin is known to have a broad range of antimicrobial activity and aucubigenin is known to inhibit cytochrome P-450, which indicates its structure could inhibit ammonia oxidisation, a key first step in nitrification, by inhibiting the enzyme ammonia monooxygenase (AMO) (Hooper & Terry 1973; Davini et al. 1986; Bartholomaeus & Ahokas 1995). Aucubin and aucubigenin likely inhibit AMO through direct binding and interaction (competitive/non-competitive inhibition) (Migneault et al. 2004; Subbarao et al. 2007). The results from Dietz et al. (2013) indicate that aucubin and its derivative aucubigenin are potential nitrification inhibitors, however, their efficacy has not been tested under ruminant urine patch conditions. The amount of aucubin entering soil through root exudation or excretion in ruminant urine has not been quantified. However, prior to quantifying aucubin inputs into soil, it is necessary to determine the inhibitory capacity of aucubin.

The aim of these studies was to determine whether plantain contained a PSM that could reduce $NO_3^-$ production, via nitrification inhibition, and $N_2O$ emissions from urine-affected soils, with particular focus on the PSM aucubin. A pilot laboratory study and subsequent field study were conducted to achieve two key objectives:

1. Determine if plantain contained a compound that inhibited nitrification and $N_2O$ emissions under urine patch conditions, and if so, determine if aucubin was the PSM responsible; and

2. Assess whether aucubin was present in plantain pasture soil and whether plantain pasture soil had reduced $NO_3^-$ concentrations and $N_2O$ emissions under urine patch conditions relative to a standard PR-WC pasture.

A laboratory study was used as a preliminary assessment of Objective 1 and a field study evaluated both Objectives 1 and 2. It was hypothesised that plantain, and its active compound aucubin, would reduce nitrification, indicated by a reduction in $NO_3^-$ accumulation, and therefore reduce $N_2O$ emissions, due to its previously identified inhibitory activity. Furthermore, it was hypothesised that plantain pasture would show reduced urine patch $N_2O$ emissions when compared to standard PR-WC pasture, due to root exudation of aucubin.

## 4.2 Materials and Methods

### 4.2.1 Laboratory trial

#### *4.2.1.1 Experimental design and treatments*

A laboratory trial was performed at Lincoln University from February 15 (Day 0) to March 15 (Day 29), 2016. The length of this trial was chosen because, under optimum conditions, nitrification is usually complete in the urine patch within 30 days (Williams & Haynes 2000; Moir et al. 2011). Soils were collected on January 22 from the Lincoln University Research Dairy Farm (43°38'32.7"S, 172°27'47.9"E) from a PR-WC pasture growing on a Paparua sandy loam soil (Typic Immature Pallic (NZ)/Udic Ustochrept (USDA)). Soil was taken to 10 cm depth, completely mixed, sieved to 4 mm, and stored at 4°C until use. The soil contained 3.9% organic matter, 2.3% total carbon, and 0.24% total nitrogen, with a C/N ratio of 9.5 (Hill Laboratories).

In the laboratory, soil (equivalent of 25 $g^{-1}$ dry weight) was added to plastic specimen vials (ThermoFisher Scientific, 70 mL volume) and incubated at 23°C with 55% humidity for 5 days before treatments were applied (Days -5 to 0). Gravimetric soil water content at Day 0 averaged 26% and soils were held at this water content for the duration of the experiment by additions of deionised water. Soils were incubated at 23°C for the duration of the experiment, as this is an optimum temperature for soil nitrification (Stark 1996; Tourna et al. 2008).

The six treatments for this trial included: Control, Urine, Urine + Plantain Leaf Extract (PLE), Urine + Aucubin Solution (AS), Urea + PLE, and Urea + AS. The PLE and AS were added to either urine or a urea solution. Urea dominates the N composition in urine and is typically 73% of the ruminant urine N deposited on pasture (Selbie et al. 2015). Urea was compared with urine since compounds in urine, other than urea, may have influenced the effects of the AS or PLE. Each of the six treatments was replicated four times, for a total of 24 vials. Six sets of these 24 vials (144 total vials) were used for this trial, with one set destructively analysed each week (Days -1, 1, 8, 15, 22, and 29).

Urine was collected on Day -1 from the Lincoln University Research Dairy Farm from cows grazing PR–WC pasture. Urine was analysed for its N content using a C–N elemental analyser (Vario–Max, Elementar GmbH), and stored at 4°C until use. Urea was dissolved in deionised water to make a urea solution with the same N concentration as the urine. Both the urine and urea solutions were applied at a rate of 700 kg N $ha^{-1}$, a typical urine patch loading rate (Selbie et al. 2015), with each vial receiving 86.5 mg N (2 mL urine or urea solution).

To make the PLE, plantain leaves (c.v. Tonic) were collected from the Lincoln University Research Dairy Farm, freeze dried, and ground to a fine powder. To extract the leaf powder, 960 mL of methanol (99%, Sigma Aldrich) was added to 48 g plantain leaf powder. The solution was placed in an

ultrasonic bath at 30°C for 1 hour and then centrifuged at 2000 rpm for 10 minutes. The supernatant was syringe filtered through a 0.45 µm filter (CA+GF, Phenex) and poured into an evaporating dish. The solution was left until all methanol had evaporated. The remaining residue was dissolved in 48 mL of deionised water and filtered through a 0.45 µm filter (CA+GF, Phenex) to produce the final PLE. The AS was prepared by dissolving 50.6 mg aucubin (Sigma Aldrich) in 10 mL deionised water. The PLE and AS were analysed for aucubin concentrations using an HPLC (Shimadzu Corporation) with a GraceSmart C18 column (250 x 4.6 mm, 5 µm) at 25°C and a mobile phase of methanol:water (10:90). Standards were purchased from Sigma Aldrich. This method was adapted from (Li & Zhao 2008). The PLE and AS were diluted as needed to produce equal concentrations of aucubin in each solution. Based on Dietz et al. (2013), aucubin was applied at a rate of 0.176 mg $g^{-1}$ dry soil (equivalent to 94 kg $ha^{-1}$), with each vial receiving 4.4 mg of aucubin (1.1 mL PLE or AS). The PLE and AS were added at the same time as the urine or urea solutions on Day 0. In the Control, 3.1 mL of deionised water was added at this time, to account for the 2 mL urine or urea solution addition and 1.1 mL PLE or AS addition to the treatment vials.

#### *4.2.1.2 Soil and gas flux sampling*

On Days -1, 1, 3, 7, 11, 14, 18, 21, 25, and 28, $N_2O$ fluxes and soil surface pH were determined. Gas fluxes were measured by placing the vials into sealable jars (283 $cm^3$ headspace) for 20 minutes (Days -1 to 21) or 10 minutes (Days 25–28), with the increase in the $N_2O$ concentration measured in the headspace every 10 (Days -1 to 21) or 5 minutes (Days 25–28) using a photoacoustic analyser (Brüel and Kjaer, Multi–gas monitor type 1302) (Kool et al. 2006b; Cayuela et al. 2010). Analyser inlet and outlet tubes were fitted with 16 gauge needles and inserted into the headspace through rubber septa fitted in the headspace chamber lid, creating a closed loop sampling system. Gas flux sampling timing was chosen based on preliminary tests of varying sampling lengths and intervals. The headspace was sealed for 20 minutes at the start of the experiment to test the linearity of the $N_2O$ emissions, and sampling time was reduced to 10 minutes when the linearity of emissions was sufficiently confirmed. Surface pH was measured using a flat–surface pH electrode (Broadley James Corporation), as explained in 3.2.2.4.

Destructive soil analyses were performed to measure soil $NH_4^+$, $NO_3^-$, DOC, and gravimetric soil moisture. Soil $NH_4^+$ and $NO_3^-$ were extracted and analysed as explained in 3.2.1, and soil $NH_4^+$-N and $NO_3^-$-N concentrations were determined using Equation 3.3. Soil DOC was extracted, analysed, and calculated as explained in 3.2.2.4. Gravimetric soil moisture was quantified using the methods in 3.2.1 and calculated using Equation 3.1.

Soil samples were analysed for aucubin concentration on Days 1, 4, and 8 by extracting 5 g (fresh weight) soil with 5 mL deionised water. Samples were shaken for 1 hr, centrifuged for 20 min at 3300 rpm, and filtered through 0.45 µm syringe filters (CA+GF, Phenex) and then frozen at -4°C. Methods for aucubin analysis were as described above (see 4.2.1.1).

As stated above (see 4.2.1.1), six sets of 24 vials (six treatments, four replicates) were used in this experiment. On Days -1, 1, 8, 15, 22, and 29, one set was destructively analysed for soil inorganic N and DOC. On each sampling day for $N_2O$ and surface pH, measurements were taken from a random remaining set, to ensure that variability among sets was accounted for. Therefore, repeated measures were not taken from one particular set of vials for these variables.

### *4.2.1.3 Data calculations and statistical analysis*

#### 4.2.1.3.1 Flux calculations

Due to technical difficulties with the photoacoustic analyser, $N_2O$ flux data before Day 7 are not presented or included in the statistical analysis. Nitrous oxide fluxes were calculated from the slope of the linear increase in concentrations within the closed chambers over the closure time. Only 53% of samples had $r^2>0.75$, however, 70% of samples with low $r^2$ values had a flux rate of less than 2 ppb $min^{-1}$, therefore low $r^2$ values are predominantly due to low flux rather than a non–linear fit. The $N_2O$ flux rate (F) was calculated using Equation 4.1 and all flux rates were corrected for air temperature during measurement and site pressure (Equation 4.2) and expressed on an elemental weight basis (µg $N_2O$–N $m^{-2}$ $hr^{-1}$).

**Equation 4.1**

$$F = \frac{b \cdot V_{CH} \cdot MW \cdot 60}{A_{CH} \cdot MV_{corr} \cdot 10^3}$$

Where:

$A_{CH}$ *:* is the surface area of the vial ($m^2$),

$b$ *:* is the increase in headspace concentration (ppb $min^{-1}$),

$MW$ : is the molecular weight of $N_2O$–N (g $mol^{-1}$),

$V_{CH}$ *:* is the volume of the headspace ($m^3$), and

$MV_{corr}$ : is the temperature corrected molecular volume ($m^3$ $mol^{-1}$) (calculated in Equation 4.2).

**Equation 4.2**

$$MV_{corr} = 0.02241 \cdot \left( \frac{273.15 + T}{273.15} \times \frac{p0}{p1} \right)$$

Where:

$T$ = the temperature during measurement (°C),

$p0$ : is the air pressure at sea level, and

$p1$ : is the air pressure at the experimental site (1 atm pressure was assumed in the calculations).

#### 4.2.1.3.2 Statistical analysis

Data were analysed using Genstat Version 18. All data had a normal distribution, tested using the Shapiro-Wilk normality test, therefore no transformations were performed. The equal variance of data was determined using the fitted-value plot. Statistical analysis was performed on data sets that met the assumptions of the ANOVA analysis. ANOVAs were performed using a 2 x 2 factorial, with [PLE vs AS] and [Urea vs Urine] as the contrasts. Least significant differences of means (5% level) were used to determine significance using Fisher's unprotected least significant difference test. ANOVAs were performed without the control, due to skewness added from this data.

Repeated measures were not taken from each vial for the duration of the experiment, as stated above (see 4.2.1.2). Therefore, cumulative $N_2O$ emissions cannot be calculated from individual replicates, as each time point does not represent the same vial. To determine differences in overall $N_2O$ emissions, an ANOVA was performed on all flux measurements over the entire experiment.

### 4.2.2 Field trial methodology

#### *4.2.2.1 Experimental site and monitoring*

The field trial was conducted from November 2 (Day 0) to December 8 (Day 36), 2016 at the Lincoln University Research Dairy Farm (43°38'27.5"S, 172°27'44.3"E) on a Paparua sandy loam soil (Typic Immature Pallic (NZ)/Udic Ustochrept (USDA)), approximately 50 m from the laboratory trial soil collection site. The field trial was established on a PR–WC pasture sown in March 2014. As stated in 4.2.1.1, this trial time length was chosen as nitrification is usually complete in the urine patch within 30 days. This trial simultaneously evaluated the first objective (the effects of PLE and AS, applied in urine, on urine patch soil inorganic N dynamics under a PR-WC pasture) and the second objective

(the effects of plantain vs PR-WC pasture on urine patch N dynamics). Field trial sites were established on a mixed sward pasture that contained 50% pure plantain (sown December 2014) and 50% pure PR-WC (sown March 2014) (Figure 4.1).

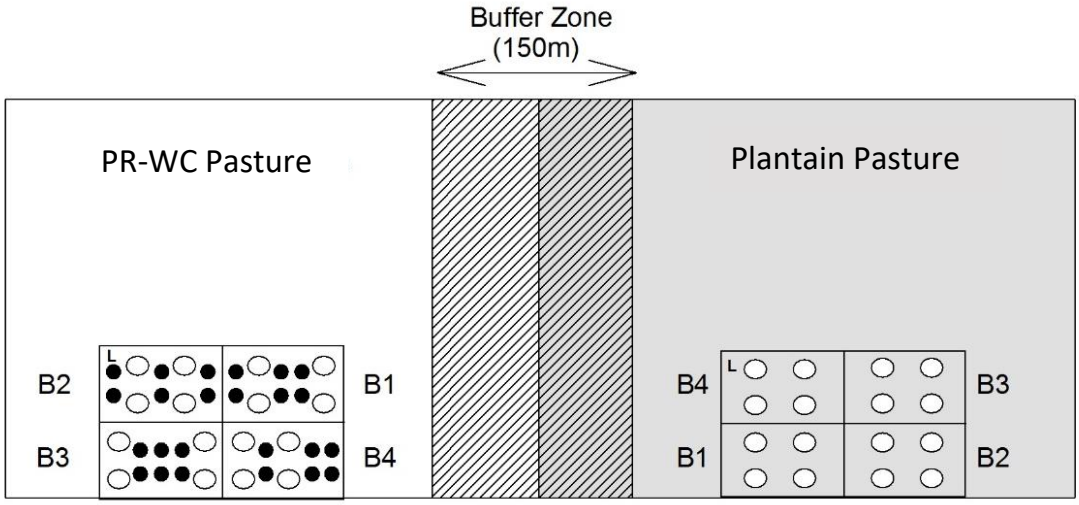



**Figure 4.1 A drawing (not to scale) of the field trial site. Trial areas are outlined and blocks are indicated by B1-B4. Each replicate within the block is represented by a pair of circles, indicating the position of the gas sampling chamber and the soil sampling area. Treatments for Objective 1, where a standard PR-WC urine was applied with either the PLE or AS present on to a PR-WC pasture, are in black. Treatments for Objective 2, where a control or standard PR-WC urine was applied to either PR-WC or plantain pasture, are in white. Logger positions are indicated by an "L" in the upper left of each pasture trial area.**

Trial sites were established >75 m to either side of the pasture transition to ensure there were no crossover plant effects. The pasture had not been grazed for at least two months prior to the experiment. Plots were irrigated with a centre pivot irrigator and an impact sprinkler to maintain the same soil water content as the laboratory trial (26% gravimetric). Pasture was cut to 5 cm before chamber installation on October 10 (Figure 4.2) and pasture height had grown to approximately 8 cm when treatments were applied.

**Figure 4.2 The plantain pasture site on the day of mowing (October 20) before chambers were installed. The site was mown from its full height (right) to 5 cm (left).**

Sensors were installed at each field site to monitor soil temperature (107, Campbell Scientific), oxygen ($O_2$) (SO–110, Apogee Instruments), and moisture (CS 616, Campbell Scientific). Soil temperature and $O_2$ sensors were installed at 10 cm depth. Each $O_2$ sensor contained a diffusive head, which integrated an area of ~385 $mm^2$ around the sensor and used a one–point calibration. The soil moisture probes were installed at an angle to integrate moisture data from 0–10 cm depth. Rainfall and irrigation were measured using a TB3 tipping bucket (1 mm) (Hydrological Services Pty Ltd.). Samples were taken every 15 minutes and data were monitored using a CR800 logger (Campbell Scientific). Air temperature, pressure, and humidity were monitored using a CR1000 logger (Campbell Scientific) and a CS215 temperature and relative humidity probe (Campbell Scientific). Each pasture contained one logger (Figure 4.1) and data were combined to give overall average experimental conditions.

**Figure 4.3 Preparing for urine treatment application onto the field site.**

A standard urine from cows grazing PR-WC pasture was used for all treatments, both Objective 1 and 2, in the field trial. Urine was collected from the Lincoln University Commercial Dairy Farm on Day -1, analysed for N content (as in 4.2.1.1), and stored at 4°C until use. Treatments were applied at 4:00pm on Day 0 (Figure 4.3).

#### *4.2.2.2 Treatments for Objective 1: Effect of PLE and AS applied in urine on PR-WC pasture*

To evaluate Objective 1, three treatments were used: Urine, Urine + PLE, and Urine + AS. Each treatment was replicated 4 times and randomised within 4 blocks. Gas sampling chamber bases (20 cm diameter) and soil sampling rings (15 cm diameter PVC pipe) were installed 7 days prior to treatment application and extended to a depth of 10 cm into the soil (Figure 4.1, Figure 4.4). The 20 cm gas chamber used for this Objective were identical to those used in 3.2.2.1, but with a 20 cm diameter rather than 40 cm. The control treatments for Objective 2 on the PR-WC pasture were also used as control treatments for Objective 1, since all chambers were randomised together.

**Figure 4.4 The 20 cm gas chamber (right) and its paired soil sampling area (left) installed on the PR-WC pasture for Objective 1.**

The PLE and AS were produced using the methods described above (see 4.2.1.1), however, a vacuum rotary evaporator was used to evaporate the methanol from the PLE using a 55°C water bath. The PLE and AS were mixed into the urine immediately before application onto the pasture and applied at the same rate of aucubin (0.088 mg $g^{-1}$ DW soil, 47 kg $ha^{-1}$), assuming 5 cm affected depth and a bulk density of 1 g $cm^{-3}$. Aucubin was applied at half the rate used in the lab trial, due to the larger scale of the trial, resulting in additions of 140 mg (93.6 mL) per gas chamber and 78.2 mg (78.2 mL) per soil sampling area. The standard PR-WC urine was applied at 700 kg N $ha^{-1}$, resulting in 0.38 L and 0.21 L of urine applied to the gas chambers and soil sampling rings, respectively.

#### *4.2.2.3 Treatments for Objective 2: Plantain vs. PR-WC pasture effects*

To evaluate Objective 2, two treatments (Control and Urine) were applied to either the PR-WC or plantain (PL) pasture. Treatments were applied to pairs of standard 40 cm diameter gas chambers, one for gas measurement and one for soil sampling. Gas chambers were identical to those used in 3.2.2.1 and were installed similarly. Each treatment was replicated 4 times and randomised within 4 blocks (Figure 4.1). The treatments applied on the PR-WC pasture were randomised within the same blocks as the chambers for Objective 1. The standard PR-WC urine was applied at 700 kg N $ha^{-1}$,

resulting in 1.4 L being added to each chamber. The control treatment received 1.4 L of water at this time.

#### *4.2.2.4 Field sampling and analysis: Objectives 1 and 2*

Nitrous oxide fluxes were monitored for 35 days after treatment application. Sampling, analysis, data transformation, and data correction followed the methods of 3.2.2.3. However, the sampling times in this study were 0, 25, and 50 min. The headspace was 10.5 L for the 40 cm diameter chambers and 2.7 L for the 20 cm diameter chambers. A predominant linear relationship was observed between the changes in headspace $N_2O$ concentration versus time (79% of all treatment samples had linear $r^2$ values > 0.90), therefore the $N_2O$ flux was determined using linear regression for all samples. Measurements with $r^2 < 0.90$ had an average Δ ppm of 0.01 inside the headspace during the 50 minute sampling, therefore low $r^2$ values are predominantly due to low flux, rather than a non-linear fit, similar to the lab trial (see 4.2.1.3.1).

Soil corers (2.5 cm diameter x 7.5 cm depth) were used to take single (20 cm chamber) or duplicate soil cores (40 cm chambers) from each soil sampling area on Days 1, 7, 14, 21, 28, and 35. Soil core samplings were spaced to avoid soil aeration effects due to previously taken cores. The duplicate cores were mixed, and all cores were stored at 4°C and extracted within 48 hours of collection. Soils were analysed for $NH_4^+$, $NO_3^-$, DOC, and gravimetric water content, according to the methods in 3.2. Additionally, 20 g soil samples were taken on Day 1 from the control treatment on plantain pasture and on Days 1-7 from the PLE and AS treatment soil sampling areas for aucubin analysis. Aucubin extraction and analysis methods were as described above (see 4.2.1.2).

Surface pH measurements were taken daily from Days 1-7, then every 2-7 days from Days 8-35, from the soil sampling areas using a flat-surface pH electrode (Broadley James Corp.), as described in 3.2.2.4.

On Days 20 and 35, hand clippers were used to cut pasture inside the gas chamber to a height of 5 cm. Pasture samples were dried at 65°C for 48 hours then weighed to determine dry matter yield (kg $ha^{-1}$). Soil sampling areas were also cut to 5 cm to replicate gas chamber conditions. Pasture DM was measured to determine whether aucubin had any unexpected significant negative effect on pasture growth inside the chamber, and is not used as an indicator of overall pasture-scale production.

On Day 36, soil cores were taken to determine soil bulk density and soil Dp/Do, as explained in 3.2.2.4.

#### *4.2.2.5 Data calculations and statistical analysis*

Cumulative $N_2O$ emissions were calculated by manually integrating the daily fluxes over time. Emission factors (EFs) were calculated by taking the difference in cumulative flux between the chamber concerned and the average cumulative flux from the control, then dividing by the total urine N applied in the given treatment.

For Objective 1, ANOVAs were performed using Genstat Version 18 with treatment and block as factors and the given measurements as the variable. Residual plots generated by the ANOVA analysis in Genstat for each variable were visually analysed to check that the data met the assumptions for the ANOVA analysis. The normality of residuals was checked using the normal plot and half-normal plot. The equal variance of data was determined using the fitted-value plot. Statistical analysis was performed on data sets that met the assumptions of the ANOVA analysis. ANOVAs were performed without the control data, due to skew added to the residual distribution from the control data. Least significant differences of means (5% level) were used to determine significance using Fisher's unprotected least significant difference test.

Statistical analysis for differences between pastures was not performed on data for Objective 2, due to having only one pasture block per treatment.

## 4.3 Results

### 4.3.1 Laboratory trial results

#### *4.3.1.1 Soil characteristics*

Soil pH in the urine or urea treated soils ranged from 7.30 to 9.56, peaking with an average value of 9.14 on Day 3 and declining to an average value of 7.71 on Day 28 (Figure 4.5). The urine– or urea–treated soils had, on average, pH values that were 2.31 units higher than the control.

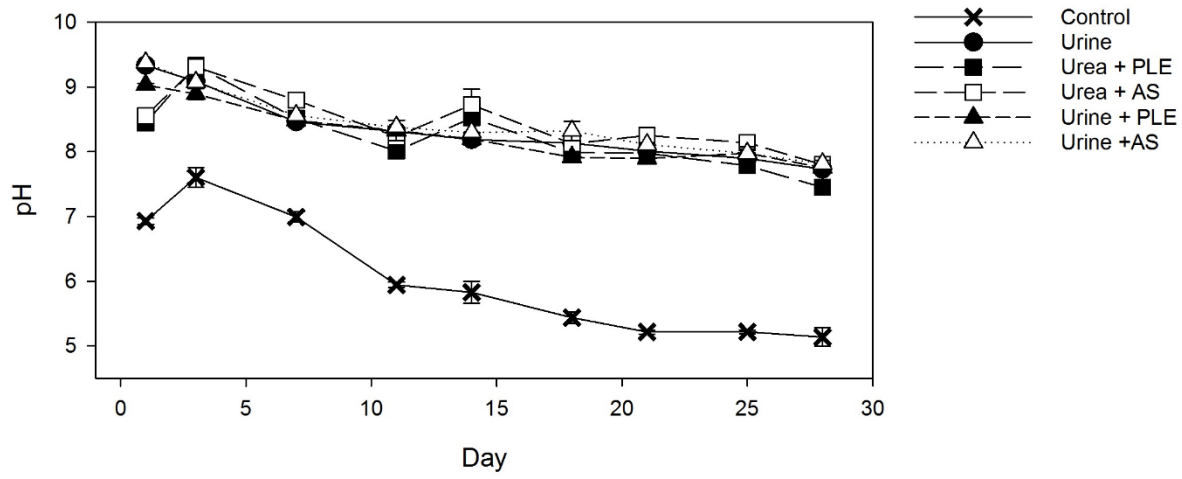



**Figure 4.5 Soil surface pH over time in the laboratory experiment (n=4). Error bars (SEM) are included on each sampling day.**

On Day 1, aucubin was detected in all PLE and AS treatments, except for the Urea + AS treatment (Table 4.1). On Day 4, aucubin was only present in the Urea + PLE treatment, and, by Day 8, aucubin was not detected in any treatment.

**Table 4.1 Aucubin remaining in the soil (mg, ± SEM) on Days 1 and 4, expressed as % applied aucubin in parentheses.**

| Treatment | Day 1 | Day 4 |
|---|---|---|
| **Urea + AS** | 0 | 0 |
| **Urea + PLE** | 3.3 ± 0.5 (74.2%) | 0.6 ± 0.1 (13.8%) |
| **Urine + PLE** | 4.0 ± 0.3 (90.4%) | 0 |
| **Urine + AS** | 3.1 ± 0.3 (69.8%) | 0 |

#### *4.3.1.2 $N_2O$ fluxes*

Fluxes of $N_2O$ averaged 4.64, 47.5, 31.4, 30.5, 34.2, and 40.0 µg $N_2O$-N $m^{-2}$ $hr^{-1}$ in the Control, Urine, Urea + PLE, Urea + AS, Urine + PLE, and Urine + AS treatments, respectively (Figure 4.6B). The average fluxes in the Urea + PLE, Urea + AS, Urine + PLE, and Urine + AS treatments were 33.8, 35.8, 28.0, and 15.6% lower than the Urine treatment, respectively. The average flux in the Urea + AS and Urea + PLE treatments was significantly lower ($P<0.05$) than the Urine treatment. There were no differences in overall averaged $N_2O$ flux between Urine vs. Urea treatments or PLE vs. AS treatments.

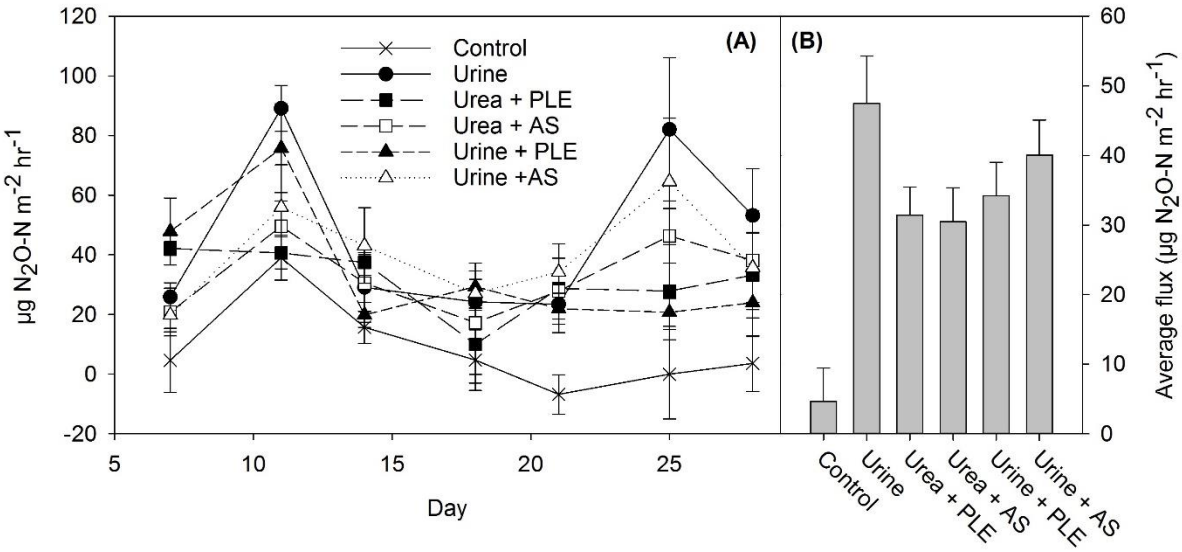



**Figure 4.6 Daily $N_2O$ flux (A) (n=4) measured in the laboratory experiment and the overall average flux for each treatment (B) (n=4) for each treatment. Error bars represent the SEM.**

There were significant differences in fluxes between urea and urine treatments on Day 18 ($P<0.05$) and between PLE and AS treatments on Day 7 ($P<0.01$; Figure 4.6A). Urine + PLE fluxes were significantly lower than Urine fluxes on Day 25 ($P<0.05$) and the Urine + AS fluxes were significantly lower than Urine fluxes on Day 11 ($P<0.05$).

### *4.3.1.3 Soil inorganic N dynamics*

On Day 1, the Urea + AS treatment contained significantly lower soil $NH_4^+$ concentrations than the other urine or urea-amended treatments ($P<0.05$). The urea treatments had higher soil $NH_4^+$ concentrations than all the urine treatments on Days 8 and 22 ($P<0.05$) while the PLE treatments contained higher soil $NH_4^+$ concentrations than the AS treatments on Days 1 and 15 ($P<0.001$; Figure 4.7A). Urea + AS had the highest $NH_4^+$ concentration on Day 29.

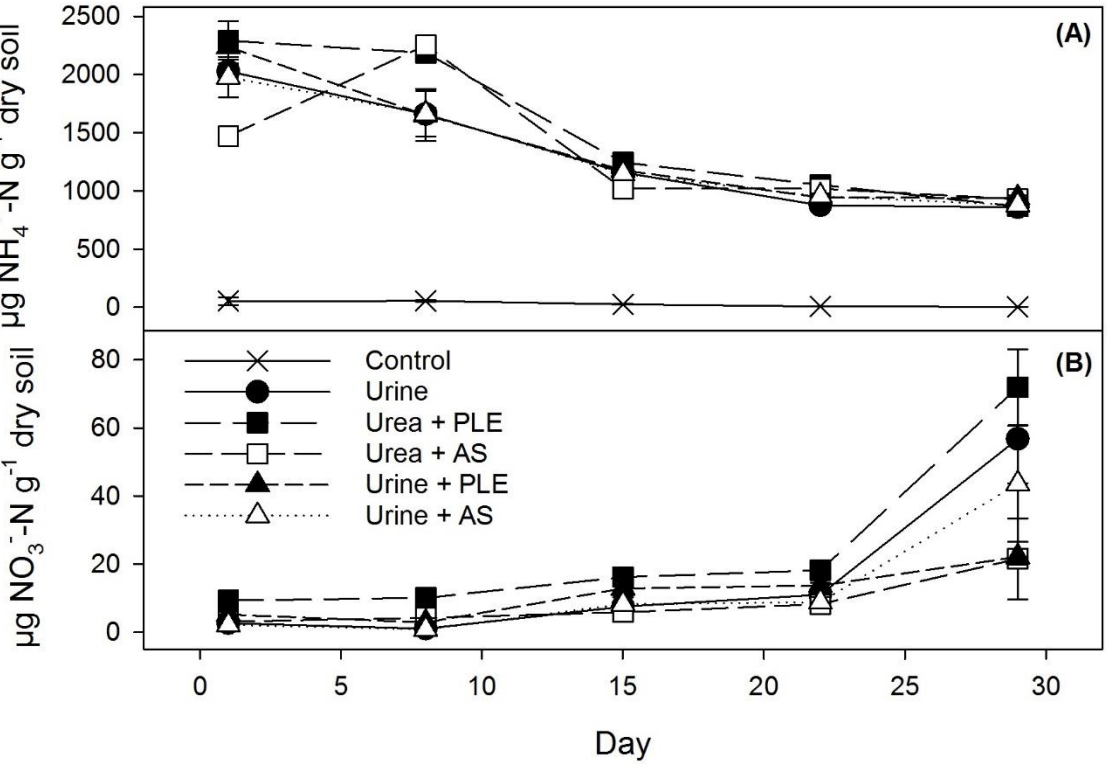



**Figure 4.7 Mean soil inorganic nitrogen concentrations ($NH_4^+$ (A) and $NO_3^-$ (B)) in the urine– or urea–treated soils for the duration of the laboratory experiment (n=4). Error bars (SEM) are included on each day.**

Regardless of treatment, the average concentrations of $NO_3^-$ concentrations remained <20 µg $NO_3^-$-N $g^{-1}$ dry soil from Days 1–22 (Figure 4.7B). The PLE treatments contained higher soil $NO_3^-$ concentrations than their AS counterparts on the first four sampling days (Days 1, 8, 15, and 22). Urea treatments had higher $NO_3^-$ concentrations than urine treatments on Days 1 and 8 ($P<0.01$) while PLE treatments had higher $NO_3^-$ concentrations than AS treatments on all days ($P<0.01$). On Day 29, $NO_3^-$ concentrations in the Urea + AS, Urine +AS, and Urine + PLE treatments were significantly lower than the Urine and Urea + PLE treatments.

### 4.3.2 Field trial results

#### *4.3.2.1 Site conditions and soil characteristics*

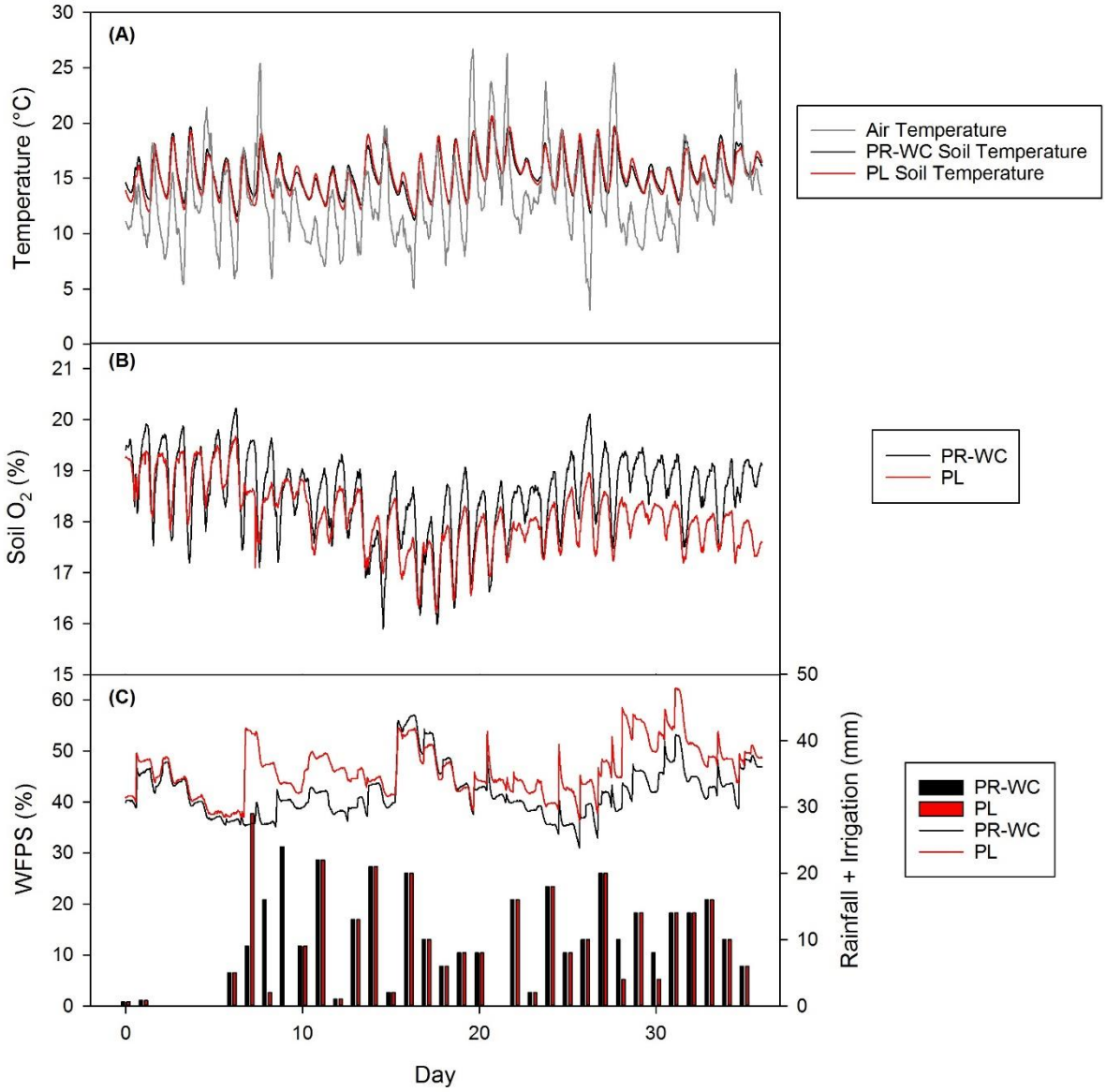



**Figure 4.8 Logger data from the two pasture sites for soil temperature (A), soil $O_2$ (B), soil WFPS (C), and total $H_2O$ inputs (C). Air temperature data was collected from the meteorological site described in 3.2.2.1. Each line represents data from one logger.**

Plots received an average of 8.1 mm rainfall or irrigation per day (Figure 4.8C). Bulk density averaged 1.17 ± 0.02 g $cm^{-3}$ in the PR-WC pasture and 1.26 ± 0.02 g $cm^{-3}$ in the plantain pasture. Water–filled pore space ranged from 31.0–57.1% and averaged 42.0% (26% gravimetric soil water content) for the duration of the experiment (Figure 4.8C), resulting in an average soil gas diffusivity of 0.051, with a range of 0.019–0.087. Air temperature, soil temperature, and soil $O_2$ averaged 13.0°C, 15.5°C, and 18.5%, respectively (Figure 4.8A,B). These averages were calculated using data from both pasture sites.

#### *4.3.2.2 Results of Objective 1: Effect of PLE and AS applied in urine on PR-WC pasture*

There were no significant treatment effects on daily $N_2O$ fluxes. Overall, $N_2O$ fluxes averaged 12.9, 283, 175, and 113 µg $N_2O$–N $m^{-2}$ $hr^{-1}$ for the Control, Urine, Urine + PLE, and Urine + AS treatments, respectively (Figure 4.9A). The cumulative $N_2O$-N flux and the EF in the Urine + AS treatment was lower than in the Urine treatment ($P<0.05$; Figure 4.9B).

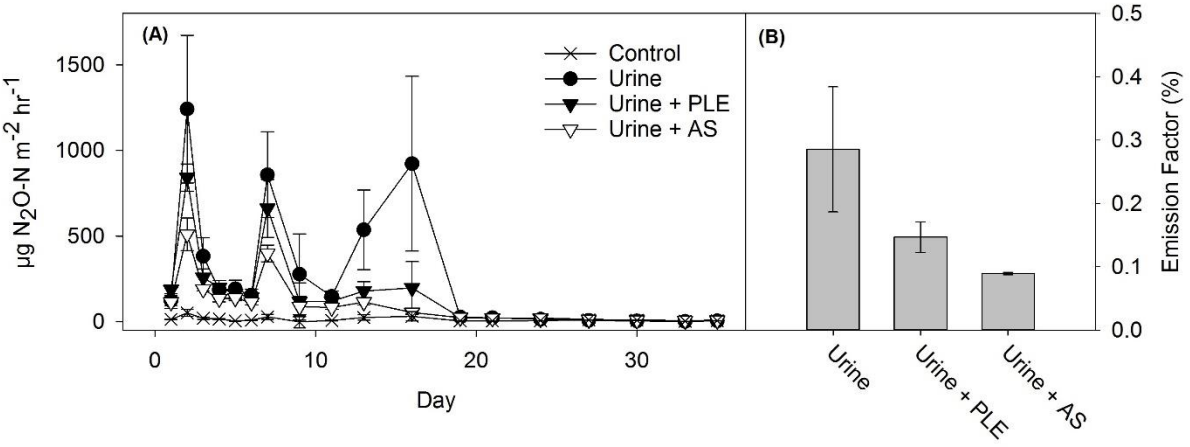



**Figure 4.9 Daily $N_2O$ flux from each treatment for the duration of the field experiment (A) and the mean EF for each treatment (B) (n=4). Error bars (SEM) are included for both the daily samples and the EF.**

The EFs averaged 0.28 (0.06-0.48), 0.15 (0.09–0.20), and 0.09% (0.09–0.10) for the Urine, Urine + PLE, and Urine + AS treatments, respectively (range in brackets). The mean EFs in the Urine + AS and Urine + PLE were reduced by 70% and 50%, respectively, compared to the Urine EF.

There were no significant treatment effects on daily or overall average inorganic N concentrations. Soil $NH_4^+$ concentrations (Figure 4.10A) peaked on Day 1 and averaged 79.1, 63.2, and 50.8 µg $NH_4^+$-N $g^{-1}$ soil in the Urine, Urine + PLE, and Urine + AS treatments, respectively. Soil $NO_3^-$ concentrations peaked on Day 21 in all treatments, with peak $NO_3^-$ concentrations highest in the Urine treatment (117 µg $NO_3^-$-N $g^{-1}$ dry soil) and lowest in the Urine + AS treatment (91 µg $NO_3^-$-N $g^{-1}$ dry soil) (Figure 4.10B).

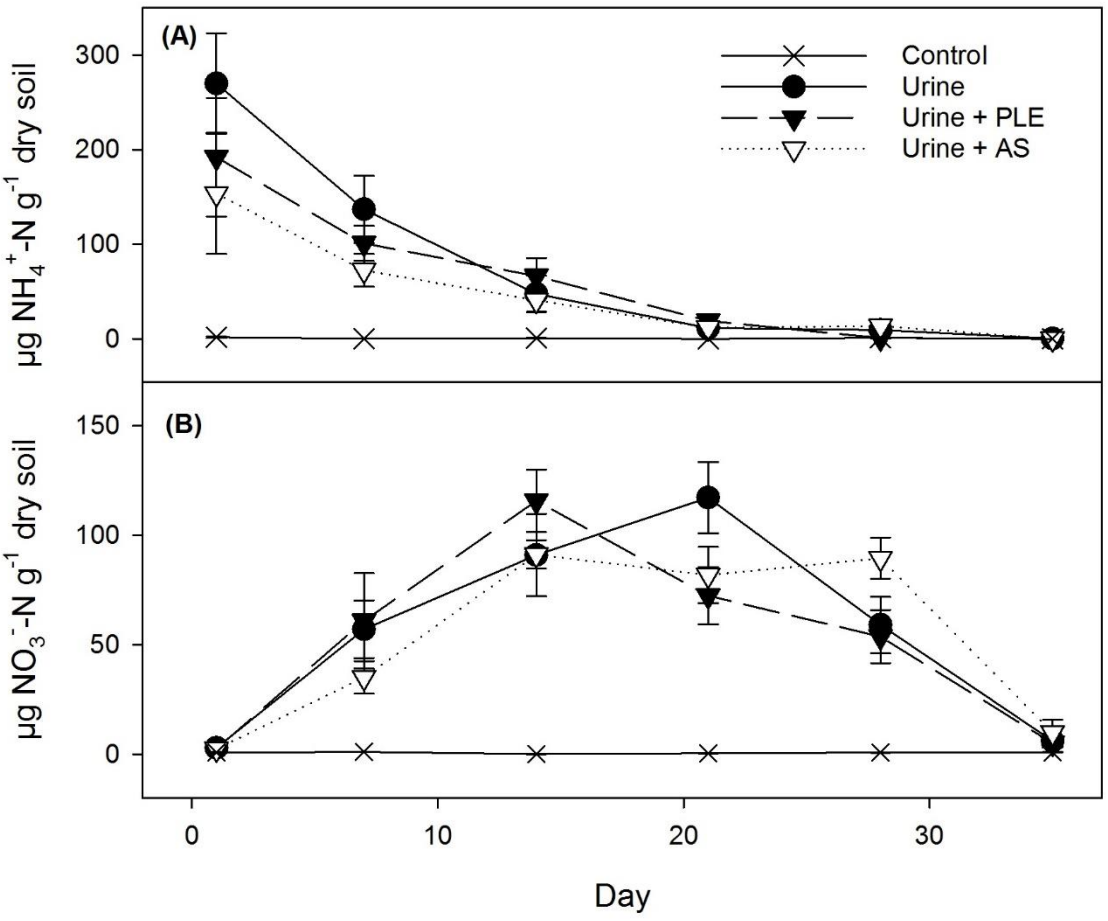



**Figure 4.10 Mean concentrations of soil $NH_4^+$ (A) and $NO_3^-$ (B) for the duration of the field experiment (n=4). Error bars represent SEM.**

Aucubin was not detected (detection limit of 0.001 mg $g^{-1}$ soil) in soils on Days 1–4, therefore no further samples were analysed for aucubin content.

There were no significant differences between treatments in total plant DM produced, which averaged 3720, 3480, and 4050 kg $ha^{-1}$ in the Urine, Urine + PLE, and Urine + AS treatments. There were also no significant differences between treatments in soil DOC concentrations, which averaged 64, 49, and 53 µg C $g^{-1}$ dry soil in the Urine, Urine + PLE, and Urine + AS treatments over the duration of the field experiment.

Overall, surface pH peaked on Day 1 and averaged 6.78, 6.63, and 6.71 for the Urine, Urine + PLE, and Urine + AS treatments, respectively (Figure 4.11).

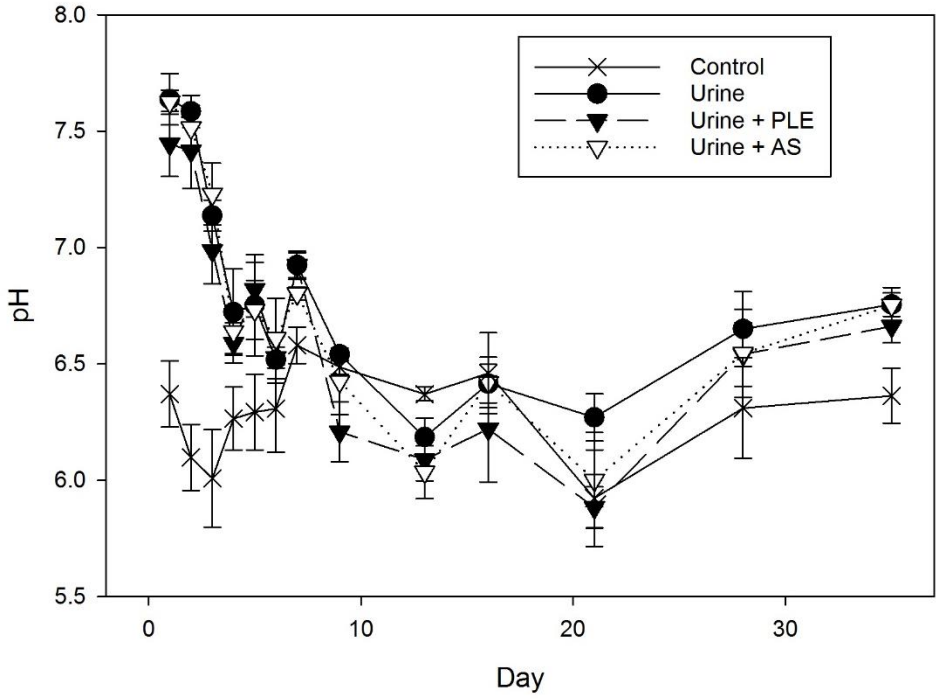



**Figure 4.11 Soil surface pH during the field experiment. Data points are means (n=4) and error bars represent SEM.**

The Urine + AS treatment had a significantly lower surface pH than the Urine treatment on Days 3 and 7, while the Urine + PLE treatment had a significantly lower pH than the Urine treatment on Days 21 and 28. The overall average pH in the Urine + PLE treatment was significantly lower ($P<0.05$) than in the Urine treatment.

#### *4.3.2.3 Results of Objective 2: Plantain vs. PR-WC pasture effects on urine N transformations*

Urine treatment $N_2O$ emissions averaged 92 µg $N_2O$-N $m^{-2}$ $hr^{-1}$ in the PR-WC pasture and 115 µg $N_2O$-N $m^{-2}$ $hr^{-1}$ in the PL pasture (Figure 4.12A). The background (control) $N_2O$ fluxes averaged 12.9 µg $N_2O$-N $m^{-2}$ $hr^{-1}$ from the PR-WC pasture and 1.2 µg $N_2O$-N $m^{-2}$ $hr^{-1}$ from the PL pasture. The EF for the PR-WC pasture averaged 0.07% (0.05-0.08%), while the EF for PL pasture averaged 0.11% (0.07-0.18%) (range in brackets) (Figure 4.12B).

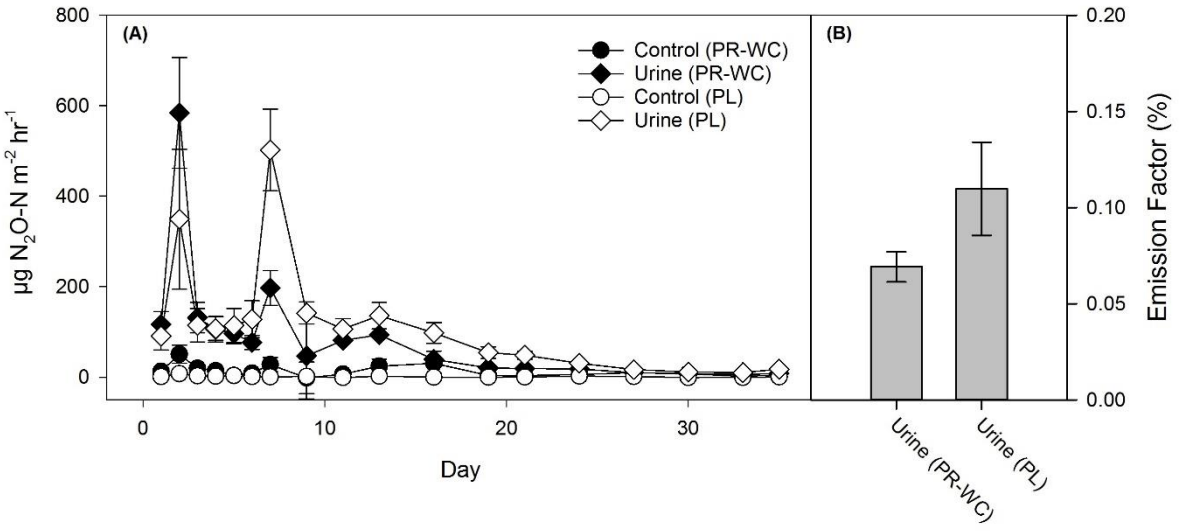



**Figure 4.12 Daily $N_2O$ fluxes (A) following the application of urine, collected from cows grazing PR-WC clover pasture, onto either PR-WC or plantain pasture (PL) and (B) the emission factor (EF) for each urine treatment. Error bars represent SEM.**

In the urine treatments, the overall average soil $NH_4^+$ concentrations were similar regardless of pasture type, averaging 117 and 81 µg $NH_4^+$-N $g^{-1}$ soil in the PR-WC and PL pastures, respectively (Figure 4.13A). Maximum soil $NH_4^+$concentration occurred on Day 1, with daily average concentrations reaching 446 and 210 µg $NH_4^+$-N $g^{-1}$ soil in the PR-WC and PL pastures, respectively.

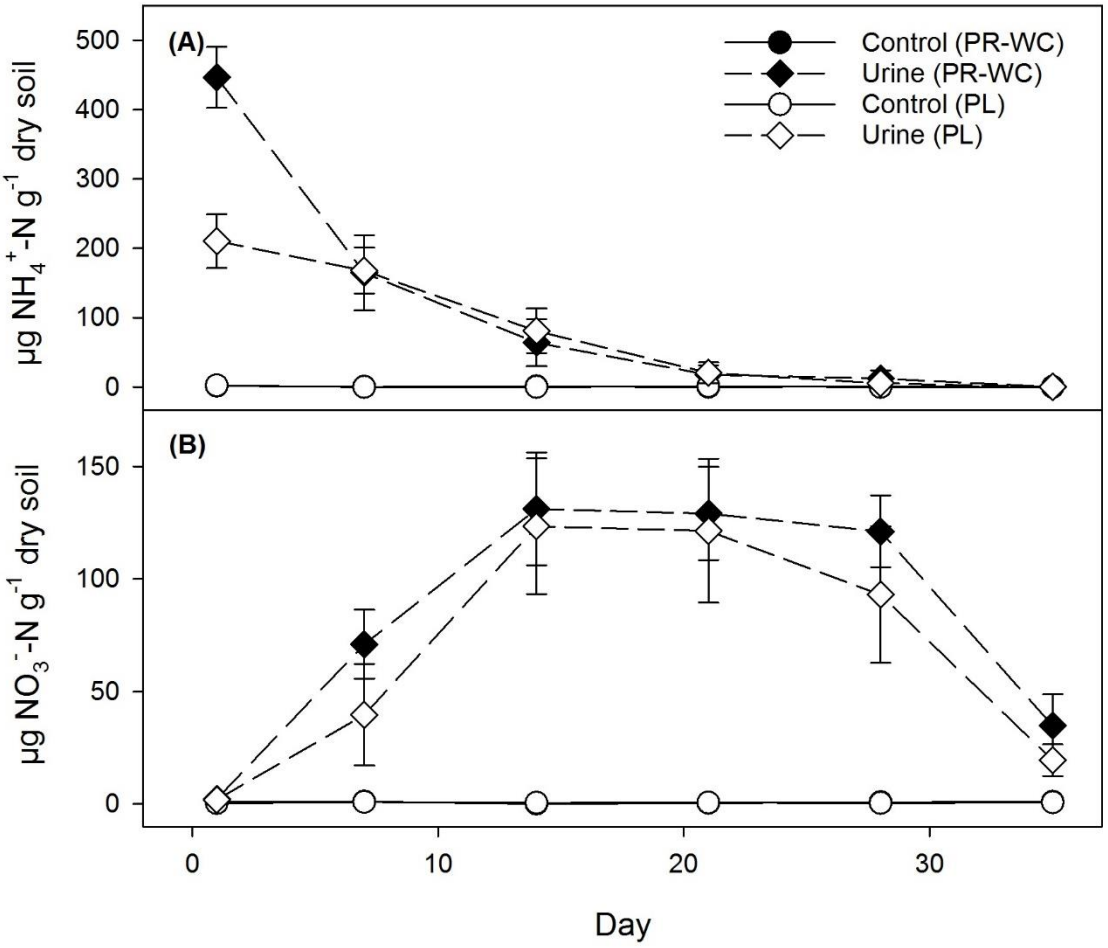



**Figure 4.13 Mean soil $NH_4^+$ (A) and $NO_3^-$ (B) over time following the application of a standard PR-WC urine onto PR-WC or PL pasture (n=4). Error bars represent SEM.**

Following urine application, the average soil $NO_3^-$ concentrations were similar under both pastures, with the PR-WC and PL pastures averaging 81.6 and 66.5 µg $NO_3^-$-N $g^{-1}$ soil, respectively (Figure 4.13B). Peak $NO_3^-$ concentrations occurred on Day 14, and reached average concentrations of 131 and 123 µg $NO_3^-$-N $g^{-1}$ soil in the PR-WC and PL pastures, respectively.

Dissolved organic carbon averaged 52.7 µg C $g^{-1}$ soil under urine in both pastures. Peak DOC reached 275 and 189 µg C $g^{-1}$ soil in the PR-WC and PL pastures, respectively.

Aucubin was not detected in the soil under the plantain pasture. The average soil surface pH was slightly higher under the PL pasture, with control and urine pH treatments averaging 6.58 and 6.71, respectively, while the respective values under the PR-WC pasture were 6.29 and 6.55, respectively. The urine treatments increased plant production by 2560 kg DM $ha^{-1}$ on the PR-WC pasture and 3080 kg DM $ha^{-1}$ on the PL pasture.

## 4.4 Discussion

### 4.4.1 Effect of AS and PLE on soil inorganic N and $N_2O$ emissions in laboratory conditions

Soil pH typically increases in the first few days following urine deposition, due to urea hydrolysis (Equation 2.1), then reduces to the pre-urine deposition alkaline state within 2-3 weeks as nitrification, a net acidifying process (Equation 2.2), occurs (Haynes & Williams 1992). However, in the laboratory experiment, soil pH remained high in all urine or urea treatments for the duration of the experiment. Additionally, approximately 40-65% of applied urine- or urea-N was present as $NH_4^+$-N on Day 1. These concentrations (~2000 µg $NH_4^+$-N $g^{-1}$ soil) are high for urine patch conditions, likely due to the lack of N removal via plant uptake. Even after the 28 day incubation period, approximately 25% of applied urine- or urea-N was present as soil $NH_4^+$-N on Day 29. With $NH_4^+$ present in elevated concentrations from the urine or urea addition, and a high soil pH as a result of urea hydrolysis, the formation of free ammonia ($NH_3$) was promoted and $NH_3$ toxicity likely occurred (van Cleemput & Samater 1995). It is known that $NH_3$ toxicity inhibits nitrification (Smith 1964; Anthonisen et al. 1976), and the lack of accumulation of soil $NO_3^-$ from Days 1-22 indicates that an almost complete inhibition of nitrification occurred. The potential inhibitory effects of aucubin were likely masked by the dominant effects of $NH_3$ toxicity until Day 29, when soil pH begins to decrease and soil $NO_3^-$ begins to accumulate.

The $N_2O$ flux measured in this study was very low, likely due to the almost complete inhibition of nitrification and $NO_3^-$ accumulation in soils throughout most of the experiment. Under field capacity soil moisture conditions, it was shown that soil $N_2O$ emissions are significantly affected by soil pH, with increasing soil pH significantly reducing soil $N_2O$ flux (Clough et al. 2004). Therefore, high soil pH measured in this experiment, as well as lack of nitrification and denitrification activity, was also not conducive to high $N_2O$ fluxes.

Even though $N_2O$ flux remained low during the study, a significant difference between the Urea + PLE and Urea + AS treatments and the Urine treatment was observed when $N_2O$ flux measurements were averaged over the entire sampling period, with overall $N_2O$ flux reduced by 33.8% in the Urea + PLE treatment and 35.8% in the Urea + AS treatment. While $N_2O$ flux was likely affected by $NH_3$ toxicity inhibiting nitrification and high soil pH, as described above, an additional inhibitory effect of the PLE and AS seems to have occurred in the urea treatments. While there was no significant difference between overall average flux in the Urea (PLE and AS) vs. Urine (PLE and AS) treatments was not significant, it seems as if the effect of aucubin as a nitrification inhibition was slightly more pronounced in the urea treatments, rather than the urine treatments, potentially due to the interaction between aucubin and the variety of compounds found in bovine urine. However, this suggested reasoning is not fully supported by the concentrations of aucubin remaining in soil over

time, since no aucubin could be extracted from the Urea + AS treatment, and the Urine + PLE treatment had the highest remaining extractable aucubin on Day 1. As explained in below in 4.4.2, aucubin can degrade to form many different compounds in soil, so the residence times measured in this experiment, which measure the residence of aucubin as the pure chemical, do not account for the residence time of the many potential degradation products of aucubin.

The Urine + AS and Urine + PLE treatments contained the highest remaining soil $NH_4^+$–N concentration on Day 29 and lowest soil $NO_3^-$–N concentration on Day 29, suggesting an inhibition of nitrification. The measured soil $NH_4^+$-N and $NO_3^-$-N concentrations were 20 and 4 times higher than the concentrations measured in Dietz et al. (2013), due to the addition of urea or urine in this experiment. Therefore, these new results extend the findings of Dietz et al. (2013) by quantifying the effects of aucubin under the high soil N concentrations prevalent in the urine patch.

The results of this laboratory experiment were hindered by the $NH_3$ toxicity that likely occurred throughout the first 3 weeks of the incubation, but the final day of soil N analysis and the significant reduction in $N_2O$ flux from the Urea + AS and Urea + PLE treatments indicate that aucubin is a potential nitrification inhibitor applied as either a water solution or plant extract, and in either urea solution or urine.

### 4.4.2 Effect of AS and PLE on soil inorganic N and $N_2O$ emissions in field conditions (Objective 1)

The field trial showed a 50 and 70% reduction in the $N_2O$ EF in the Urine + PLE and Urine + AS treatments, respectively, when compared to the Urine treatment, with the EF significantly reduced in the Urine + AS treatment. Emissions of $N_2O$ were low in the second half of the field trial (Days 19–35), likely because the aerobic conditions were not conducive to denitrification. However, soils were intentionally kept aerobic to promote nitrification, which was the focus of the experiment. The EFs of all treatments are within the normal range for urine patches (Selbie et al. 2015).

Soil inorganic N was not significantly different between treatments on any given day and no other measured variables showed overall significance in the study. Furthermore, aucubin was not found in soils on Day 1. This differs with what was observed in the laboratory study, possibly because of the lower aucubin application rate or possibly due to a potentially increased aucubin degradation rate under field conditions. Aucubin is known to be most stable in solution at pH 10 (Chun & Cho 1995), therefore the higher soil pH values in the laboratory trial, compared with the field trial, may explain why aucubin remained in soils longer. It is also known that different fungal strains degrade plantain PSMs at different rates (Gonda et al. 2013), meaning that the intact fungal communities in situ could

have significantly affected the residence time of aucubin in soil. Furthermore, in solution, aucubin is known to degrade completely to its unstable aglycone, aucubigenin, in the presence of β–glucosidase within four hours (Kim et al. 2000) and β–glucosidase is known to be present in soils (Busto & Perez-Mateos 1995; Busto & Perez-Mateos 2000). Aucubigenin can be further transformed to an unsaturated aldehyde, which can irreversibly bind to nucleophilic side chains of nucleic acids and proteins, due to its strong alkylating properties (Kim et al. 2000; Marak et al. 2002). Aucubin can also degrade to catalpol, another plantain PSM, or its aglycone (Marak et al. 2002). Therefore, the degradation products of aucubin were likely still present in soils during the experiment, however, the soil analysis only measured aucubin remaining as the pure compound. The large number of possible structures make it difficult to determine its exact fate or residence time in each form. Further studies are required to examine the breakdown products of aucubin in soils, particularly aucubigenin, which is the known active antimicrobial degradation product (Bartholomaeus & Ahokas 1995). Additional measurements of soil nitrifier activity would help determine if and when nitrifiers are inhibited by aucubin or its degradation products.

### 4.4.3 Plantain vs. PR-WC pasture effects on soil inorganic N dynamics and $N_2O$ emissions (Objective 2)

The $N_2O$ (both daily flux and EF), $NH_4^+$, and $NO_3^-$ data from the PL pasture and PR-WC pasture did not indicate significant differences in nitrification rates between the two soils, however, as stated in 4.2.2.5, no statistical analysis was performed on this data due to the lack of multiple-pasture replication (n=1 for each treatment). Although the average $N_2O$ flux for the Urine treatment on the plantain pasture was higher than the Urine treatment flux on the PR-WC pasture (115 vs. 92 μg N $m^{-2}$ $hr^{-1}$, respectively), the Control emissions were much lower from the plantain pasture compared to the PR-WC pasture (1.2 vs 13 μg N $m^{-2}$ $hr^{-1}$, respectively). The average $N_2O$ flux data can be used to calculate average pasture-scale emissions during the 5 weeks after a grazing event, assuming that each grazing period results in 5.8% of pasture covered with urine (Moir et al. 2011). Therefore, 5.8% of the pasture would produce the Urine treatment emissions, while the remaining 94.2% of the pasture would produce the Control treatment emissions. Using these calculations, following a grazing event, the average $N_2O$ flux from plantain pasture at a paddock-scale would be 7.8 μg $N_2O$-N $m^{-2}$ $hr^{-1}$, while the average flux from the PR-WC pasture at a paddock-scale would be 17.6 μg $N_2O$-N $m^{-2}$ $hr^{-1}$. Although the plantain pasture produced higher $N_2O$ emissions under the Urine treatment, it produces a lower calculated paddock-scale $N_2O$ flux due to its low measured background emissions.

It is known that plantain exudes PSMs (including aucubin and catalpol) from its roots, and that the presence of microorganisms and nematodes increase the concentration of aucubin in plantain roots and root exudation (Wurst et al. 2010). However, it was not possible to extract aucubin from the

plantain pasture soil. This could be due to the many forms to which aucubin can degrade, as stated above (see 4.4.2), or due to the conditions in grazed pasture soil. Some plants have developed mechanisms, such as excretion of nitrification-inhibiting PSMs, to increase N use efficiency and minimise N losses, since N is a limiting nutrient in many ecosystems (Rice & Pancholy 1972; Subbarao et al. 2006). However, intensive dairy farming systems frequently apply N fertiliser to maintain high pasture production (Parfitt et al. 2012), therefore plants that exhibit these characteristics, such as plantain, could potentially show reduced excretion of inhibitory chemicals under intensive dairy pasture conditions due to the abundance of available N. It has been shown that plantain grown under low nutrient conditions had higher aucubin and catalpol concentrations in the roots compared to plantain grown under high nutrient conditions (Darrow & Bowers 1999). Therefore, the grazed pasture system may not promote PSM excretion from plantain roots.

### 4.4.4 Rate of aucubin application onto soil

The rate of aucubin entering soil, either through excretion in ruminant urine or exudation from plantain roots, remains unknown. One study has been performed to analyse the effect of aucubin on rumen degradation, and found that aucubin reduces in vitro ammonia production and is likely degraded to its active aglycone aucubigenin in the rumen (Navarrete et al. 2016). However, this study did not measure the residence time of aucubin and/or aucubigenin, or determine its fate after ruminal digestion, therefore, it remains unknown in what form or at what concentration aucubin is excreted in ruminant urine. It is known that aucubin is present in roots and can comprise 1-2% of plantain root DM (Darrow & Bowers 1999), but the rate of aucubin exudation from plantain roots, or the form of aucubin present in plantain pasture soils, has not been determined. Therefore, aucubin application rates used in this study were based on previous concentrations that showed significant reductions in $NO_3^-$ accumulation (Dietz et al. 2013). However, as stated above, that experiment was not performed under urine patch conditions, and soil $NH_4^+$ and $NO_3^-$ concentrations in this experiment were much higher. Therefore, it is possible that aucubin application rates need to be increased to produce a significant reduction in $NO_3^-$ accumulation in the urine patch.

Aucubin was applied at a rate of 47 and 94 kg $ha^{-1}$ in the field trial and laboratory trials, respectively. However, the urinary excretion of aucubin could potentially be 486 kg $ha^{-1}$ (assuming 100% excretion of consumed aucubin, 18 kg dry matter intake $day^{-1}$ $cow^{-1}$ (Box et al. 2016), 1% aucubin content in dry matter (Darrow & Bowers 1999; Wurst et al. 2010), 10 urine events per day (Selbie et al. 2015), and a urine patch size of 0.37 $m^2$ (Moir et al. 2011)). It has been shown that aucubin can reach concentrations of 7% in plant dry matter (Fuchs & Bowers 2004), which would increase potential aucubin excretion to 3,405 kg $ha^{-1}$, 72 and 36 times higher than the rates used in the field trial and

lab trial, respectively. Although these calculated values represent the upper limit of potential aucubin excretion, they show that there is the potential for aucubin to be present in urine patches at concentrations much higher than used in these studies, which could potentially increase the degree of nitrification inhibition. Future studies should test the aucubin application rate needed to produce significant decreases in both $NO_3^-$ concentrations and $N_2O$ emissions from the urine patch, and determine whether these concentrations are found in ruminant urine or plantain root exudates.

## 4.5 Conclusions

The results from the laboratory study indicated that aucubin, applied in a water solution or in a plant leaf extract to the urine patch, could potentially reduce soil nitrification and decrease average $N_2O$ flux after urine deposition. The similarity between PLE and AS treatment results in the laboratory study supported the hypothesis that aucubin was the active PSM in plantain that inhibited nitrification in the presence of urea or urine. The field trial (Objective 1) produced similar $N_2O$ flux results as the laboratory trial, with a reduction in the mean $N_2O$ EF of up to 70% ($P<0.05$), but soil inorganic N data did not indicate that nitrification inhibition occurred. It remains unclear whether aucubin or its derivatives are present in plantain pasture soil and whether this leads to any differences in soil inorganic N dynamics or $N_2O$ emissions (Objective 2). It is possible that plantain root exudation of PSMs explains the lower background emissions measured from the plantain pasture, which resulted in an overall lower calculated pasture-scale $N_2O$ flux after an assumed "typical" grazing event. A repeat of the experiment conducted for Objective 1 is warranted due to the variability in $N_2O$ emissions measured during this experiment, and should include more replicates per treatment and larger chamber sizes. This repeat experiment was performed and is described in Chapter 5.

# Chapter 5
# Reassessing the potential for aucubin to reduce urine patch $NO_3^-$ accumulation and $N_2O$ emissions under field conditions

## 5.1 Introduction

Nitrous oxide ($N_2O$) is a potent greenhouse gas (GHG) and significant contributor to New Zealand's overall GHG emissions (Ravishankara et al. 2009; Ministry for the Environment 2016). It has been estimated that 94% of New Zealand's $N_2O$ emissions originate from grazed grasslands, driven by grazing livestock urine deposition (Ministry for the Environment 2015a). Therefore, mitigation strategies to reduce $N_2O$ emissions from urine patches are needed. Nitrification inhibitors have proven to significantly reduce urine patch $N_2O$ emissions, but they are not currently used in New Zealand, due to issues with their implementation (e.g. chemical transfer into milk) (Di & Cameron 2002a, 2003; Moir et al. 2007; Di & Cameron 2012; Cameron et al. 2014; Di & Cameron 2016). Therefore, the identification of novel nitrification inhibitors is required for future GHG mitigation strategies.

A novel biological nitrification inhibitor, aucubin, was evaluated in the two studies summarised in Chapter 4. Aucubin is a plant secondary metabolite (PSM) found in plantain, a pasture herb species used in New Zealand, and is recognised as having antimicrobial activity, with inhibitory activity on soil nitrification previously identified (Davini et al. 1986; Bartholomaeus & Ahokas 1995; Dietz et al. 2013; Massaccesi et al. 2015). A review of aucubin as a nitrification inhibitor was given in 2.5.2.3. The results reported in Chapter 4 were inconclusive, due to the variability in the field trial $N_2O$ data, with inconsistencies between the results of the two experiments (e.g. a significant reduction in soil $NO_3^-$ in the laboratory trial but not in the field trial). Therefore an additional study was warranted to further evaluate the efficacy of aucubin as a nitrification inhibitor.

The additional study, presented in this chapter, was performed in the field to evaluate the effects of aucubin *in situ*, as these are the conditions in which real urine patch $N_2O$ emissions occur. The results in Chapter 4 indicate that aucubin is the active PSM in plantain, therefore an aucubin treatment, but no plantain leaf extract treatment, was used in this follow-up experiment. This field trial was run at a similar time of year to the field trial performed in Chapter 4, so that conditions would be relatively similar between the two trials. Soil water-filled pore space (WFPS) was maintained at ~50%, since it has been shown that nitrification is the dominant source of $N_2O$ at this WFPS (Bateman & Baggs 2005). The same rate of aucubin application, as used in Objective 1 of Chapter 4, was used in this trial, which is equivalent to ~10% of the calculated highest potential aucubin excretion rate (see 4.4.4).

It was concluded in 4.4.2 that further analytical methods for determining the breakdown products of aucubin, and their residence times in soil, and microbial response to aucubin application were needed for future experiments. However, these methods were not included in this secondary trial due to the budgetary constraints of this PhD research programme. It is still recommended that future experiments develop and include these techniques and measurements to monitor the degradation and inhibitory capacity of aucubin in soil.

Four key differences between this trial and the previous field trial in Chapter 4 were:

1. Larger chamber bases (40 cm diameter) were used to increase the sampling area per chamber, with the goal of reducing variability between chambers;

2. The number of replicates for each treatment were increased from 4 to 6;

3. The trial was performed on a site that was previously maintained with 'cut-and-carry' mowing, rather than grazing, to eliminate any possible background effects of previous urine patches; and

4. Urine N loading was reduced from 700 kg N $ha^{-1}$ to 500 kg N $ha^{-1}$, as this loading rate has recently been identified as being more typical of the urine N loading from cows grazing plantain as a proportion of their diets (Di et al. 2016; Woods 2017).

It was hypothesised that adding aucubin to ruminant urine would reduce urine patch $N_2O$ emissions due to aucubin acting as a nitrification inhibitor. The inhibitory capacity of aucubin was assessed using measurements of soil inorganic N, soil pH, and soil $N_2O$ flux to monitor the progression of soil nitrification and the resulting $N_2O$ emissions.

## 5.2 Methods

### 5.2.1 Trial site preparation

The field site was established at the Lincoln University Field Research Centre (43°38'51.4"S, 172°28'01.6"E) on a PR-WC pasture. The pasture had not been grazed for over one year, with routine cutting and removal of forage. The site area was mown before chamber installation on September 4, 2017. To monitor $N_2O$ emissions, 18 gas chambers (40 cm diameter) were installed in a randomised block design, with 6 blocks. The chambers were identical to those used in 3.2.2.1.

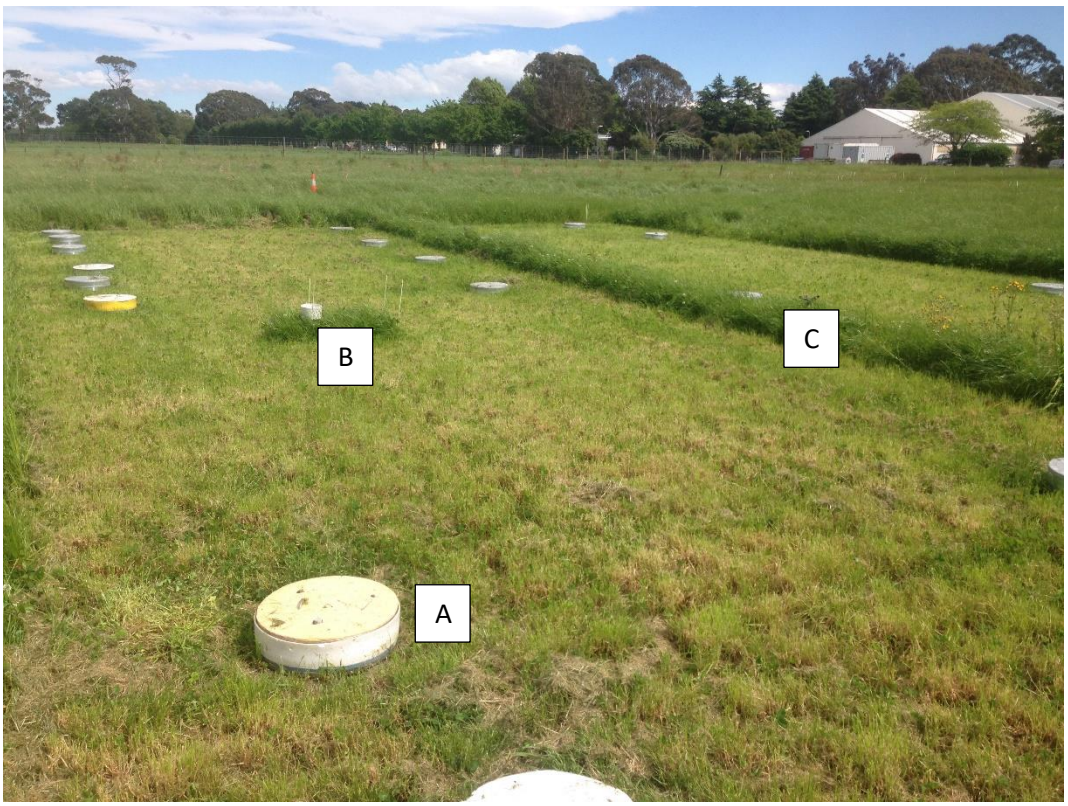



**Figure 5.1 The field site, showing the closed gas sampling chambers (A), logger site (B), and centre line irrigation (C). The irrigation pipe is concealed by the long grass.**

A centre line pivot irrigator was installed through the middle of the sampling site, with 3 sampling blocks on either side (Figure 5.1). A logger (CR200x, Campbell Scientific) was installed at the field site, with a soil moisture sensor (Decagon 5TE, Decagon Devices), and a Pronamic rain gauge (300.023-20) to measure irrigation and rainfall. Data were logged hourly and down-loaded via telemetry and an intra web interface (Campbell Scientific Loggernet Admin and Vista Data Vision). Air and soil temperature data were also collected from the NIWA meteorological station described in 3.2.2.1, which was 100 m from the current field site.

Soil BD was determined according to the methods of 3.2.2.4. Soil WFPS was calculated using VWC data obtained from the soil moisture sensor along with soil BD, using the methods of 3.2.1 and Equation 3.2.

The area inside the gas chamber was cut to 5 cm on September 19 and on October 2 (Day -2) using electric hand shears and all cut plant matter was removed from inside the chamber. Treatments were applied on October 4, 2017 (Day 0) and sampling occurred until November 8 (Day 35).

### 5.2.2 Treatment preparation and application

Treatments included: Control, Urine, and Urine + Aucubin. Each treatment was replicated 6 times. Urine was collected on October 2 from the Lincoln University Research Dairy farm from cows grazing PR-WC pasture. The urine was analysed for N content, as described in 4.2.1.1, and stored at 4°C until use. In the Urine + Aucubin treatment, aucubin was applied at 47 kg $ha^{-1}$ (544 mg per chamber), equivalent to the rate used in 4.2.2.2. Aucubin was purchased from Nanjing Leading Chemical Co. (Nanjing, China). Aucubin was dissolved directly into the urine, rather than dissolved into water and then applied into urine, as was done in Chapter 4. The Urine + Aucubin treatment was applied within 30 min of dissolving the aucubin into the urine. Treatments were applied at 11:00 a.m. on October 4, with urine applied at a rate of 500 kg N $ha^{-1}$ (0.91 L per chamber), which is more typical of the urine N loading rate of a cow grazing plantain as a proportion of their diet (Di et al. 2016; Woods 2017).

### 5.2.3 Gas, soil, pH, and plant measurements

Nitrous oxide sampling, analysis, and flux calculations were performed according to the methods in 3.2.2.3 (see Figure 5.2). The $N_2O$ samples were also analysed for $CO_2$ concentrations using the automated gas chromatograph system described in 3.2.2.3. The $CO_2$ was reduced to $CH_4$ using a methaniser, and C concentrations were quantified with a flame ionisation detector, with a detection limit of 150 $\mu L\ L^{-1}$. Fluxes of $CO_2$ were determined in a similar manner to the $N_2O$ fluxes and corrected using the methods of Venterea (2010), as described in 3.2.2.3. Surface pH was measured as explained in 3.2.2.4. Above-ground plant biomass was cut to 5 cm above ground level on Day 34, and processed as previously described (3.2.2.4) to determine total DM production.

**Figure 5.2 An open gas chamber before gas sampling commenced. The gas sampling syringe and pre-evacuated 6 mL Exetainers for sample collection can be seen in the red tray. Inside the gas chamber, 50 mL falcon tubes fill the holes where soil cores were taken, as explained in the text.**

One soil sampling core (2.5 cm diameter x 10 cm depth) was taken from inside the gas sampling chamber each week. Cores were taken after gas sampling was performed, so that the soil profile was not disturbed before gas sampling. Soil samples were taken from inside the gas chamber, rather than from a separate soil sampling area, due to the extremely high cost of purchasing aucubin for treatment application. A 50 mL plastic falcon tube (Nest Biotechnology Co. Ltd.) was inserted immediately into the area where the soil core had been removed. The tube completely filled the hole and reduced the effects of $O_2$ diffusion into area around where the core had been removed. Over the experiment, 5 cores were removed, with each core representing 0.4% of the soil surface area. Therefore, the total removed surface area inside the gas chamber was ~2%. A sixth soil core was

taken on Day 35 after the final gas sampling. The soil cores were extracted and analysed according to the methods of 3.2.1 to determine inorganic N content and gravimetric water content.

### 5.2.4 Statistical analysis

All data analyses were performed using Genstat Version 18. Data were analysed using ANOVA, with treatment and block as factors. Residual plots generated by the ANOVA analysis in Genstat for each variable were visually analysed to check that the data met the assumptions for the ANOVA analysis. The normality of residuals was checked using the normal plot and half-normal plot, and tested using the Shapiro-Wilk normality test. The equal variance of data was determined using the fitted-value plot. Statistical analysis was performed on data sets that met the assumptions of the ANOVA analysis. Significance was determined using least significant differences of means and Fisher's unprotected least significant difference test. The control data were not included in statistical analysis for any measured variable, due to skew added to the distribution of residuals from the control data. Daily $N_2O$ and inorganic N data were log transformed to meet the assumptions of normality. Both the raw and log transformed data for $N_2O$ flux and soil inorganic N are presented, but statistical significance is only presented for the log transformed data for these variables. Plant DM data and soil surface pH data were not transformed as both data sets were normally distributed.

Cumulative $N_2O$ emissions were calculated by manually integrating both the raw daily fluxes and the log transformed daily fluxes over time. Emission factors (EFs) were calculated by taking the difference in the raw cumulative $N_2O$-N flux between the treatment chamber concerned and the average raw cumulative $N_2O$-N flux from the control, then dividing by the total urine N applied. Statistical analysis was performed on the EFs calculated from the raw data so that this value could be compared to previous chapters. Additionally, analyses were performed on the cumulative log transformed $N_2O$-N emissions, to account for skew in the raw daily flux data, and on the cumulative log transformed $N_2O$-N emissions from Day 0-10, which represents the initial peak flux period for this trial.

## 5.3 Results

### 5.3.1 Experimental site conditions

Soil BD averaged 1.23 g $cm^{-3}$ over all blocks, and did not vary significantly between blocks. The air temperature during the 35 day experiment ranged from 2.2-25°C, with an average temperature of 12°C (including both day and night temperatures) (Figure 5.3A). The soil surface temperature averaged 13°C and the soil temperature at 5 cm averaged 14°C. The temperature data was collected

from the meteorological site described in 5.2.1. Rainfall and irrigation application averaged 3 mm per day, with the highest rainfall event occurring on Day 30 (32.4 mm). The experimental site received 107 mm of rainfall and irrigation over the sampling period (Figure 5.3B). Soil WFPS ranged from 39-57% and averaged 50% over the entire experiment (Figure 5.3B).

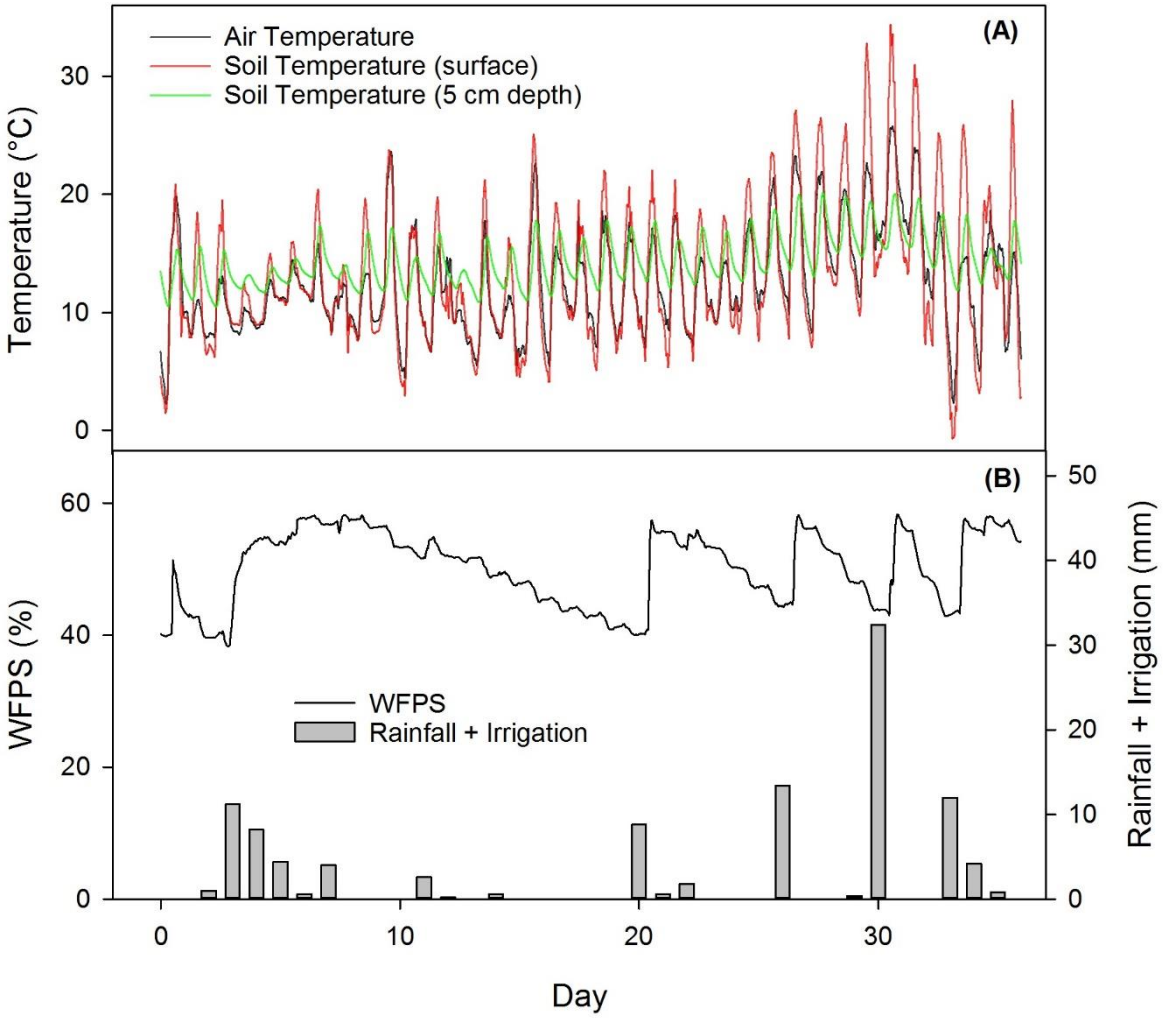



**Figure 5.3 Site conditions over the 35 day experiment. Temperature data (A) were obtained from the meteorological station near the field site, as described in 5.2.1, while rainfall data were collected from the logger installed at the field site (B).**

### 5.3.2 Nitrous oxide and carbon dioxide emissions

Overall, $N_2O$ flux averaged 172 µg $N_2O$-N $m^{-2}$ $h^{-1}$ in the Urine treatment and 101 µg $N_2O$-N $m^{-2}$ $h^{-1}$ in the Urine + Aucubin treatment (Figure 5.4A). Treatment EFs, calculated from the raw flux data, averaged 0.22% in the Urine treatment and 0.13% in the Urine + Aucubin treatment, and were not significantly different from each other (Figure 5.4B).

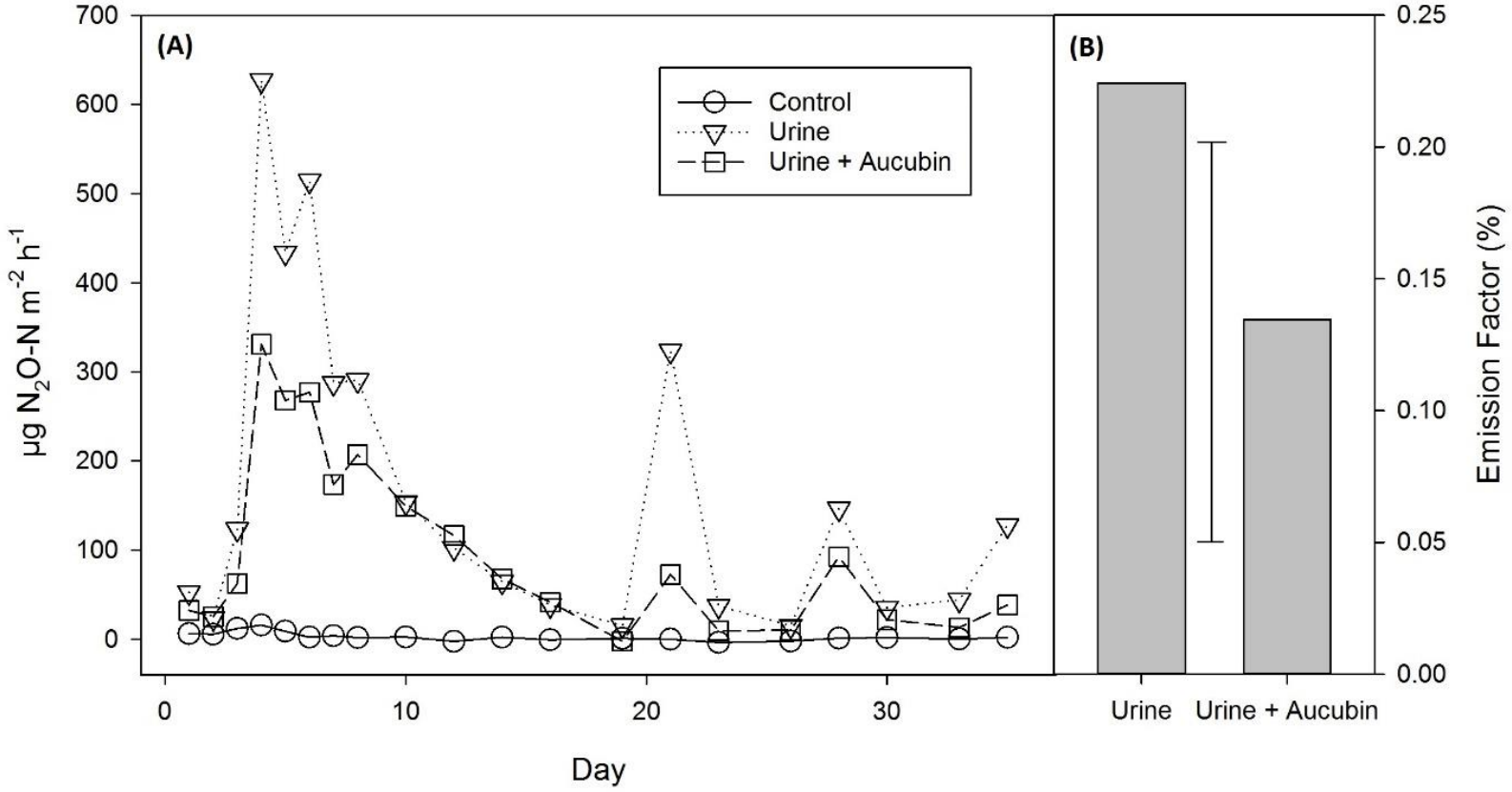



**Figure 5.4 Mean raw $N_2O$ flux (n=6) for the three treatments in the field trial (A) and the calculated emission factor for the two urine treatments (B). Error is reported as LSD (5%) for the EFs, but no error is reported for the daily flux because no statistical analysis was performed on this raw data.**

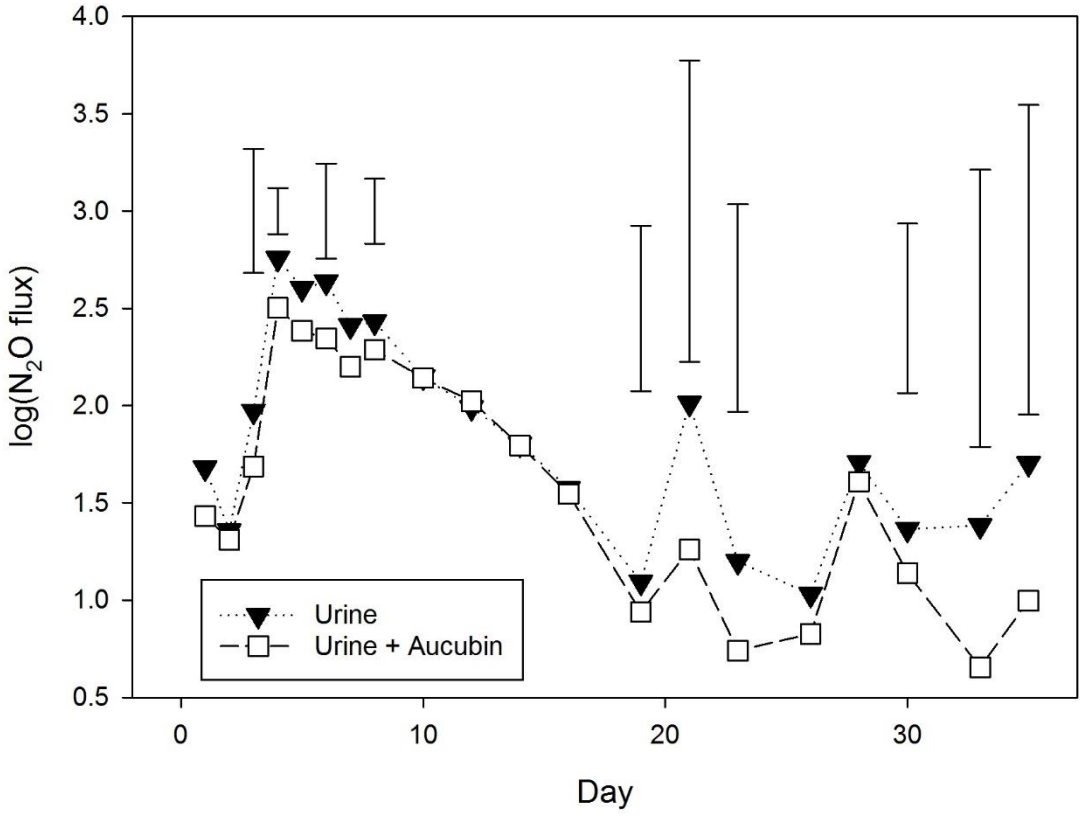



**Figure 5.5 Log transformed mean daily $N_2O$ flux (n=6), reported with LSD bars (5%) on some days to give an indication of overall variability.**

Following log transformation of the data, the daily $N_2O$ flux was significantly higher in the Urine treatment compared to the Urine + Aucubin treatment on Day 4 ($P<0.05$), but did not differ on any other day (Figure 5.5). Cumulative emissions from Day 0-10, indicating the initial flux period, did not differ between treatments. Similarly, cumulative emissions summed over all 35 days did not differ between treatments.

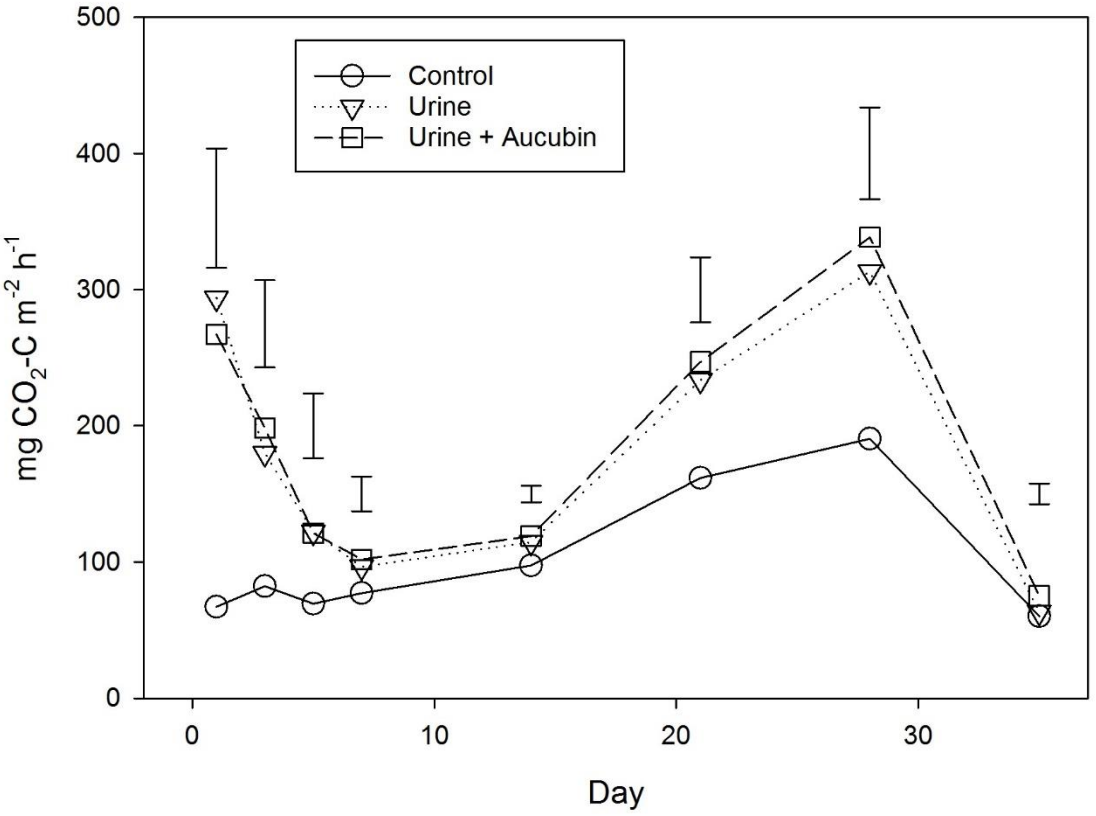



**Figure 5.6 Soil $CO_2$-C emissions from the three treatments over the 35 day experiment. Data points are means (n=6), with error reported as LSD (5%). Error was calculated using only the Urine and Urine + Aucubin treatments because the control was not used for statistical comparisons. Above-ground plant biomass was cut for sampling on Day 34 and the final $CO_2$ sampling was taken on Day 35.**

Peak $CO_2$ emissions occurred on Day 28 in all treatments, reaching 190, 310, and 340 mg $CO_2$-C $m^{-2}$ $h^{-1}$ in the Control, Urine, and Urine + Aucubin treatments, respectively (Figure 5.6). Differences in $CO_2$ fluxes between the two urine-amended treatments were not significant on any day, and average fluxes over the 35 day experiment were not significantly different between the two treatments.

### 5.3.3 Soil and plant measurements

Soil $NH_4^+$ concentrations increased rapidly in both treatments, with individual chambers reaching maximum concentrations of 86 µg $NH_4^+$-N $g^{-1}$ dry soil in the Urine treatment on Day 7 and 70.8 µg $NH_4^+$-N $g^{-1}$ dry soil in the Urine + Aucubin treatment on Day 1 (Figure 5.7).

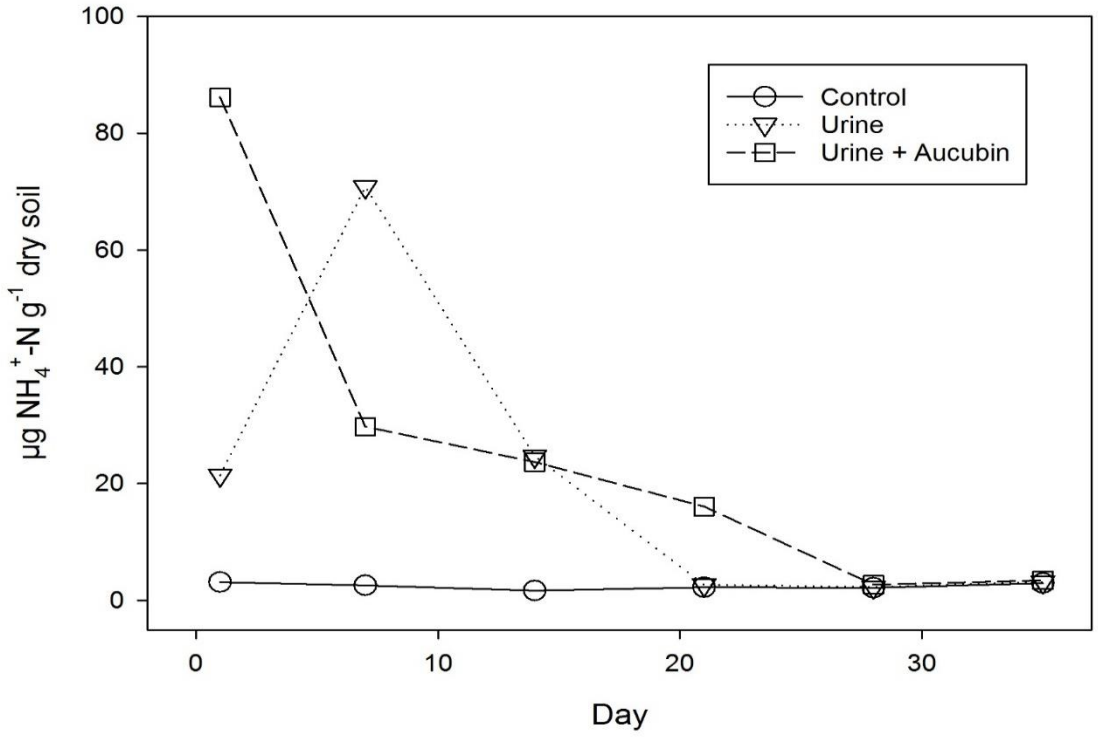



**Figure 5.7 Mean raw soil $NH_4^+$ data (n=6) for the three treatments in this experiment. No error bars are included because no statistical analysis was performed on this data due to the data being skewed.**

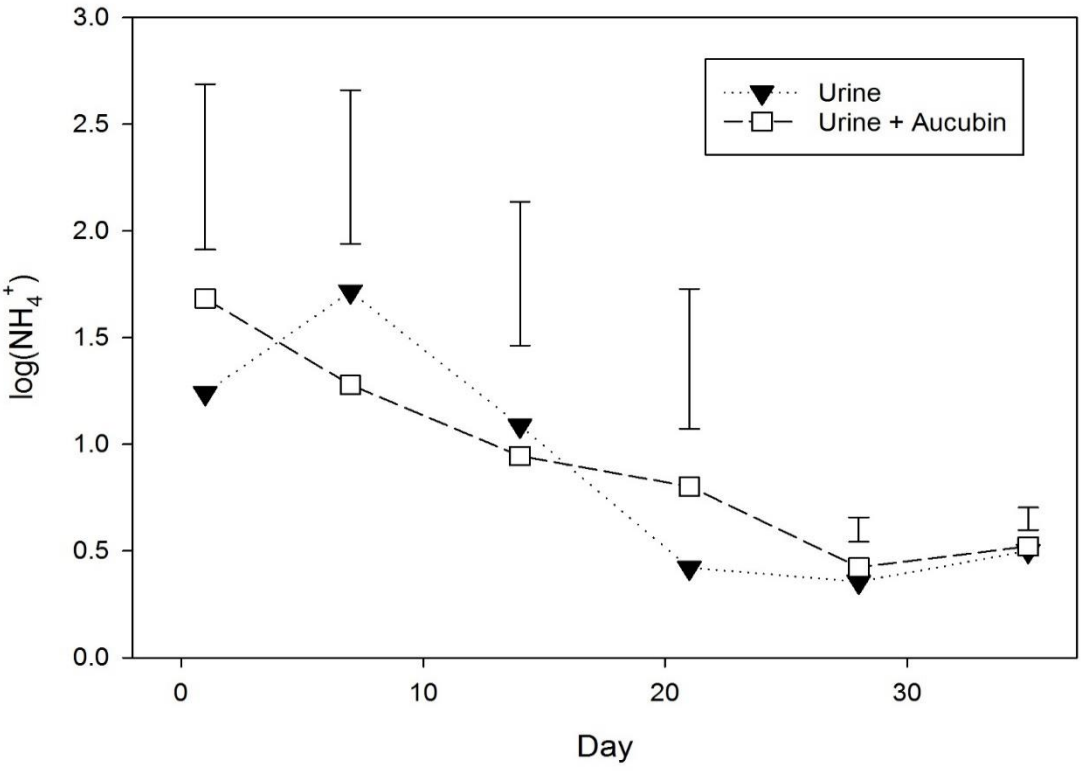



**Figure 5.8 Log transformed soil $NH_4^+$ on each sampling day, with LSD bars (5%) for each sampling day (n=6).**

Soil $NH_4^+$ concentrations in both treatments returned to baseline levels by Day 28. Overall, soil $NH_4^+$ averaged 2.5, 21, and 27 µg $NH_4^+$-N $g^{-1}$ dry soil in the control, Urine, and Urine + Aucubin treatments, respectively (Figure 5.7). No significant differences in soil $NH_4^+$ concentrations occurred between treatments on any sampling day, or when averaged over the entire experiment (Figure 5.8).

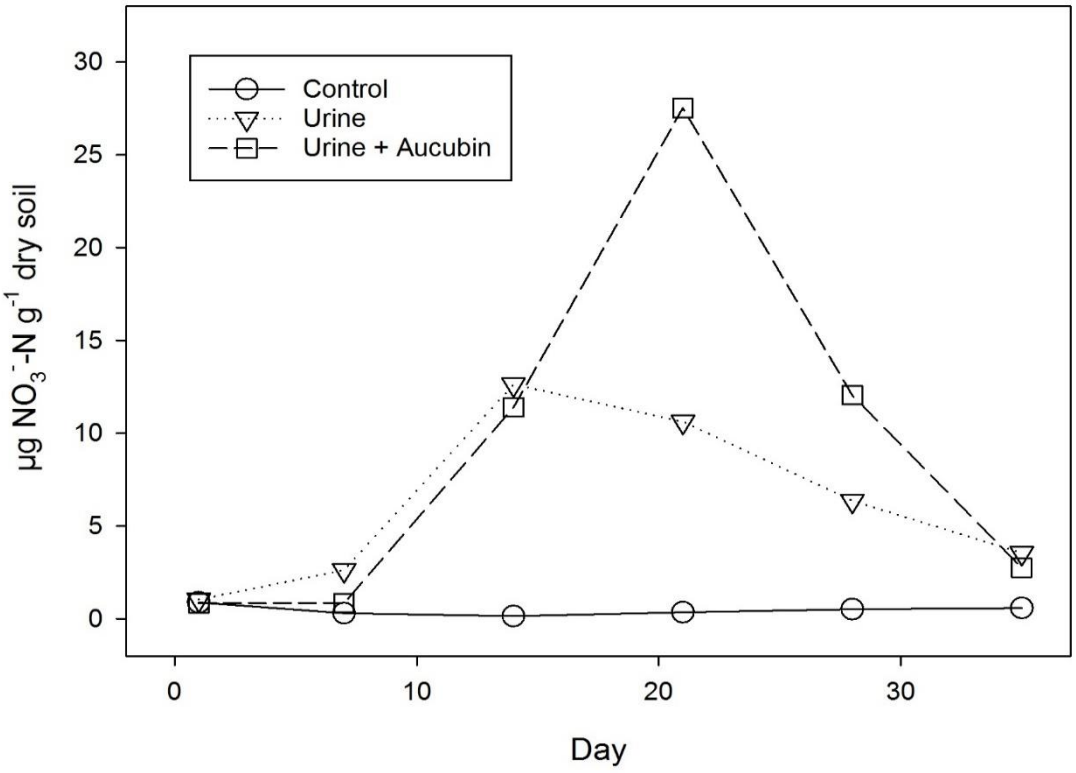



**Figure 5.9 Mean raw soil $NO_3^-$ data (n=6) for the three treatments in this experiment. No error bars are included because no statistical analysis was performed due to raw data being skewed.**

Soil $NO_3^-$ concentrations peaked on Day 14 and 21 in the Urine and Urine + Aucubin treatments, respectively, and overall averaged 6.2 and 9.2 µg $NO_3^-$-N $g^{-1}$ dry soil in the two treatments, respectively (Figure 5.9). The control averaged 0.46 µg $NO_3^-$-N $g^{-1}$ dry soil during the experiment.

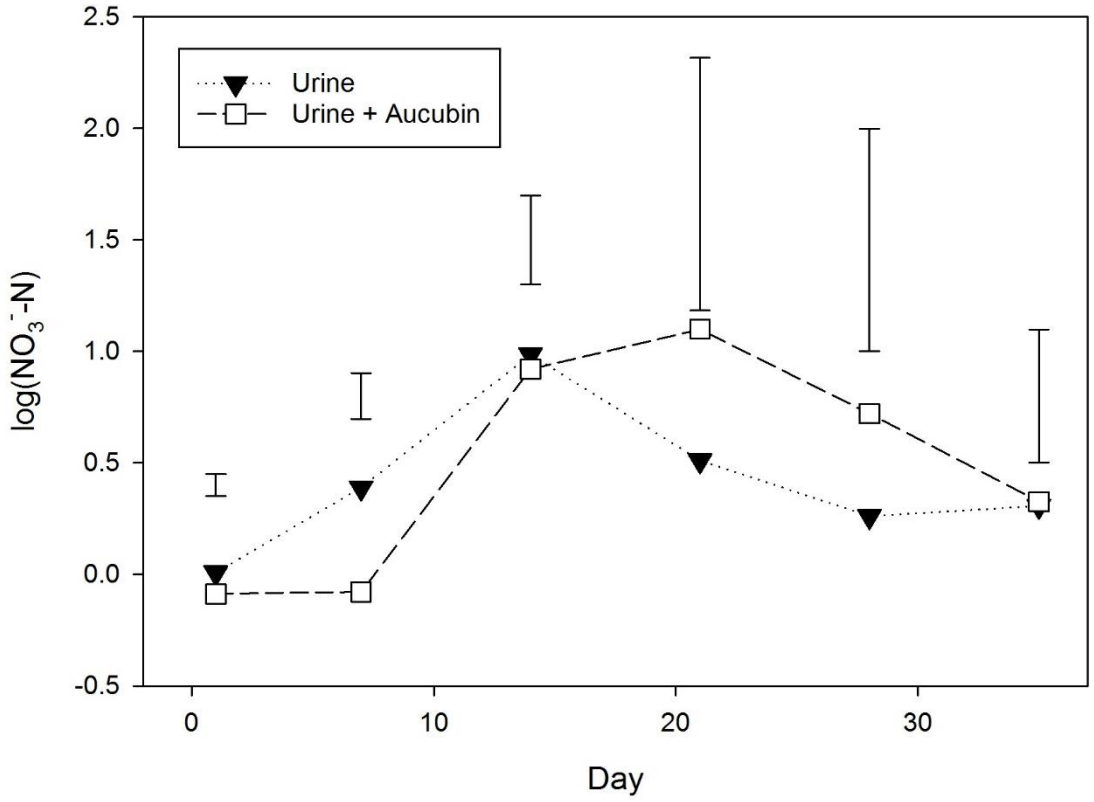



**Figure 5.10 Log transformed soil $NO_3^-$ on each sampling day, with LSD bars (5%) for each data point (n=6).**

The soil $NO_3^-$ concentration in the Urine treatment was significantly higher than in the Urine + Aucubin treatment on Day 7 ($P<0.05$), but no other significant differences between soil $NO_3^-$ concentrations occurred on the other sampling days (Figure 5.10). Additionally, when soil $NO_3^-$ concentrations were averaged over the experimental period, there was no significant difference between treatments.

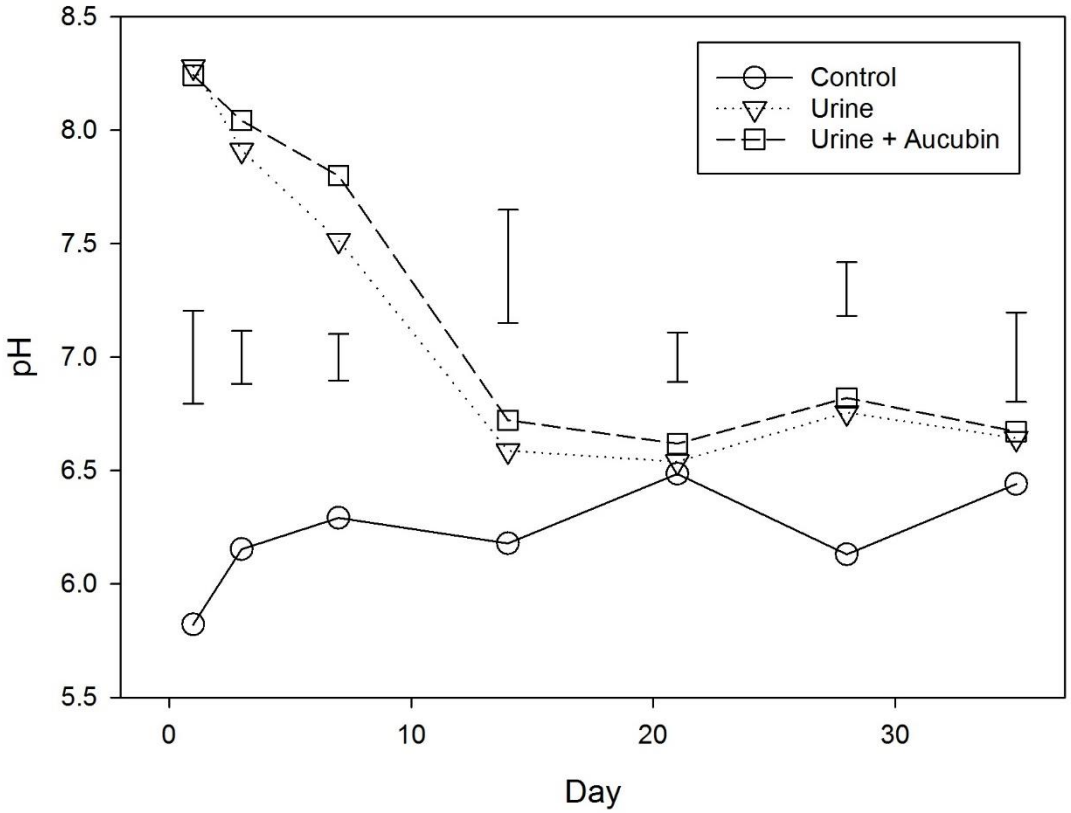



**Figure 5.11 Soil surface pH for each treatment (n=6) over the experiment. Error bars (LSD; 5%) are included on all days and were calculated excluding the control treatment.**

Soil surface pH averaged 6.21, 7.17, and 7.27 in the control, Urine, and Urine + Aucubin treatments, respectively (Figure 5.11). The surface pH in both urine-amended treatments peaked on Day 1. Surface pH was significantly higher in the Urine + Aucubin treatment than the Urine treatment on Day 7 ($P<0.05$), but was not significantly different on any other sampling day. When averaged over all sampling days, the surface pH in the Urine + Aucubin treatment was significantly higher than the Urine treatment ($P<0.05$).

Total plant DM production over the 35 day trial averaged 2190, 3820, 3730 kg DM $ha^{-1}$ in the Control, Urine, and Urine + Aucubin treatments, respectively. There was no significant difference in plant DM production between the Urine and Urine + Aucubin treatments.

## 5.4 Discussion

### 5.4.1 Nitrous oxide emissions

Contrary to our hypothesis, the $N_2O$ EF was not significantly reduced when aucubin was added to the urine patch. While the mean $N_2O$ flux in the Urine + Aucubin treatment was lower on all but 4 sampling days, there was only one day where the treatments were significantly different, resulting in

an overall lack of difference between the EFs. This result is similar to the trends in the $N_2O$ flux data in Objective 1 of the Chapter 4 field trial (see 4.3.2.2), which is the field trial that this field trial was based on. This previous trial is henceforth referred to as O1C4. In O1C4, the Urine treatment produced a higher mean $N_2O$ flux than the Urine + Aucubin treatment on most days, but, similarly, these differences were not significant.

The higher urine N loading rate (700 kg N $ha^{-1}$) in O1C4 is the likely to be the reason for the higher $N_2O$ flux observed in that trial, but the EFs, the percentage of N emitted as $N_2O$-N, were similar in both the Urine and Urine + Aucubin treatments between O1C4 and the present trial. The experiments were performed at similar times of the year to enable the comparison of results under similar site conditions (e.g. soil moisture and temperature), therefore it was expected that EFs would be comparable between the trials, as these are key factors influencing $N_2O$ emissions (Firestone & Davidson 1989). As stated in 4.4.2, these EFs are within the normal range for urine patches (Selbie et al. 2015).

In both trials, soil $NH_4^+$ peaked after urine deposition, and returned to approximately pre-urine deposition levels, indicating that nitrification was complete. Therefore, all $N_2O$ emissions derived from nitrification were captured during the experimental period. Similarly, $NO_3^-$ concentrations had declined to be close to control levels and thus continued $N_2O$ emissions beyond the sampling period, as a result of the treatment application, would likely have been low, due to low $NO_3^-$ availability as substrate for denitrification.

### 5.4.2 Differences in soil N availability compared to previous field trial (O1C4)

Using the urine N application rate, soil BD, and assuming soil was affected by urine N infiltration to a 10 cm depth, we can calculate that the urine N loading rate per g soil in this experiment (406.5 µg N $g^{-1}$ soil) and in O1C4 (598.5 µg N $g^{-1}$ soil). The peak $NH_4^+$ concentrations only reached ~60-80 µg $NH_4^+$-N $g^{-1}$ soil in this trial, which is lower than typical urine patch conditions (Haynes & Williams 1993; Selbie et al. 2015), but the reasons for this are not clear, since conditions, such as soil moisture and temperature, were similar between experiments, as explained below.

Similar trends occurred between the two experiments with respect to the soil $NH_4^+$ concentrations in the Urine + Aucubin treatments, and in soil $NO_3^-$ concentrations in both the Urine and Urine + Aucubin treatments. As stated above, the abiotic conditions, such as temperature and soil moisture, were similar between the two experiments, so it is not likely that these factors contributed to the difference in soil N availability. Since soil moisture conditions were relatively aerobic in both experiments, $NO_3^-$ leaching loss and gaseous $N_2$ loss were also likely to have been similar between

experiments. Soil pH trends were similar between experiments, with soil pH increasing after urine application, then decreasing to return to background levels by the end of the sampling period. Additionally, the plant DM production and $N_2O$ EFs were similar between the experiments, meaning that N loss via those pathways was similar as well.

### 5.4.3 Indicators of a general inhibitory effects of aucubin on soil microbes

Soil $CO_2$ flux provides a measure of overall soil microbial activity, since soil microbial populations could not be assessed due to budgetary constraints. The lack of difference in $CO_2$ flux between the Urine and Urine + Aucubin treatments suggest that, overall, microbial populations were not significantly inhibited by the presence of aucubin at a rate of 47 kg $ha^{-1}$. Aucubin is a known active antimicrobial inhibitor (Davini et al. 1986), with inhibitory mechanisms hypothesised to be similar to that of glutaraldehyde (Bartholomaeus & Ahokas 1995), but the lack of difference in $CO_2$ flux between treatments indicates that the concentration used in this experiment may not have been high enough to produce a significant inhibitory effect on overall soil microbial activity. However, the aim of this experiment was not to assess aucubin as a general microbial inhibitor, but rather to assess it as a nitrification inhibitor.

Similar to O1C4, there were no differences soil $NH_4^+$ concentrations between the Urine and Urine + Aucubin treatments on any day. The raw $NH_4^+$ data appears to show a difference in the rate of $NH_4^+$ formation, via urea hydrolysis, with soil $NH_4^+$ concentrations peaking in the Urine treatment on Day 1 and peaking in the Urine + Aucubin treatment on Day 7. These trends are typical of urease inhibition (Zaman & Nguyen 2012), and therefore indicate that aucubin may have had an inhibitory effect on urease activity. However, the differences in the soil $NH_4^+$ concentrations between the two treatments were not significant, therefore we cannot conclude that aucubin had any effect on urease activity. Additionally, these differences in $NH_4^+$ peaks may be due to a matter of timing, since the length of time between soil sampling dates means that any peak in soil $NH_4^+$ between Days 1 and 7 was not accounted for.

A similar trend was observed in the laboratory trial of Chapter 4 (see Figure 4.7), where soil $NH_4^+$ was significantly lower in the Urea + AS treatment than the Urine treatment on Day 1 and did not peak until Day 7 in this treatment. However, this was the only treatment, out of the four PLE or AS treatments, which showed this trend.

### 5.4.4 Aucubin as a nitrification inhibitor

While aucubin did not show signs of being a general microbial inhibitor or urease inhibitor, the $N_2O$, soil $NO_3^-$, and surface pH data, when considered as a whole, indicate that aucubin inhibited nitrification for a short period around Days 4-7. Since $N_2O$ is produced via the intermediates of nitrification (Firestone & Davidson 1989), the significantly lower $N_2O$ emissions on Day 4 indicate that nitrification, and the production of nitrification intermediates, was occurring at a slower rate in the Urine + Aucubin treatment. The significantly lower $NO_3^-$ concentration and significantly higher surface pH in this treatment on Day 7 further suggest that nitrification was inhibited, as $NO_3^-$ is a product of nitrification, and soil pH is reduced during nitrification (see 2.3.3.4). No differences in soil $NH_4^+$, the substrate for nitrification, were observed between treatments, but it is important to note that $NH_4^+$ soil concentration is a net measure, and factors such as plant uptake, immobilisation, mineralisation or $NH_3$ loss also significantly affect soil $NH_4^+$ concentrations (Haynes & Williams 1993).

This short period of inhibitory activity is similar to the results found in both the laboratory trial and field trial in Chapter 4, where aucubin was found to degrade rapidly in soils (4.3.1.1, 4.3.2.2). As the aglycone of aucubin, aucubigenin, is the known active product of aucubin (Bartholomaeus & Ahokas 1995), it is likely that aucubin is degraded in soils within 4 days, as observed in the Chapter 4 laboratory trial, and that aucubigenin remains active in soils for approximately 3 days, based on the length of the period that differences in $N_2O$ emissions, soil $NO_3^-$, or soil surface pH were observed in this current experiment.

However, this inhibitory period is much shorter than in Dietz et al. (2013), where application of aucubin at a similar rate led to an almost complete inhibition of soil $NO_3^-$ accumulation for 56 days. There are differences in experimental conditions which may have led to the difference in inhibitory activity:

1. Soil moisture: When in solution, glutaraldehyde undergoes rapid hydration, and can exist as a mixture of hydrated forms, in equilibrium (Migneault et al. 2004). This may also occur when aucubin is dissolved in urine, or in the soil solution, as these compounds are hypothesised to react similarly (Bartholomaeus & Ahokas 1995). Additionally, under anaerobic conditions, the dialdehyde form of aucubigenin can react with free $NH_3$ or $NH_4^+$ to form pyridine monoterpene alkaloids, such as aucubinine (Hattori et al. 1990; Yang et al. 2003). Aucubin was added to soils as a solution in this experiment, and in Dietz et al. (2013). However, the soil moisture conditions were different between the two experiments: Dietz et al. (2013) maintained soil moisture at 70% WHC, while this experiment was performed at approximately 100% WHC (-10 kPa).

2. Soil pH: Soil pH increases in urine patch conditions (Haynes & Williams 1992), and pH is known to affect the fate and reactivity of aucubin (Ghisalberti 1998) and glutaraldehyde (Migneault et al. 2004), a compound which reacts similarly to aucubin. Aucubin is most stable at pH 10 (Kim et al. 2000), and acids can degrade aucubin to aucubigenin, the active aglycone of aucubin. Therefore, the lower pH in Dietz et al. (2013) may have converted aucubin to its aglycone, aucubigenin, which then actively inhibited nitrification.

3. No leaching loss: Rainfall and irrigation were applied to this field trial, and aucubin may have been removed from the system via leaching. Dietz et al. (2013) performed their study under laboratory conditions, with no leaching loss from the system.

However, the most likely reasoning for the difference in inhibitory activity of aucubin between this experiment and Dietz et al. (2013) is the difference in ratio of aucubin to ammonia oxidising bacteria (AOB) populations. It is known that nitrification is driven by AOB activity under elevated concentrations of urea, such as under urine patches, and that the addition of urine can increase AOB populations by 177-fold (Di et al. 2009; Prosser & Nicol 2012; Samad et al. 2017). Since aucubin has been hypothesised to inhibit microbial activity via cross-linking (Bartholomaeus & Ahokas 1995), there must be sufficient concentrations of aucubin to react with the AOB present to result in a significant reduction in soil $NO_3^-$ accumulation. It is likely that the concentrations of aucubin used by Dietz et al. (2013) were at high enough ratios to soil AOB populations to significantly reduce nitrifying activity, but these same levels of aucubin application in the current experiment may have resulted in a significantly lower ratio of aucubin to soil AOB populations, since soil AOB populations were likely to have increased greatly due to the urine application.

Thus, it is likely that the rate of aucubin application needs to be increased under urine patch conditions, compared to the rate applied in Dietz et al. (2013), to produce a similar level of significance in nitrification inhibition.

## 5.5 Conclusions

Similar to the results of Chapter 4, there are indications in the soil $N_2O$ and inorganic N data that aucubin acts as a nitrification inhibitor, but the overall results of the experiment do not provide statistically significant evidence that aucubin, when applied to soil, is a potent inhibitor with lasting effects. It is likely that aucubin is degraded rapidly in soils, and that the inhibitory effects of its degradation products, particularly aucubigenin, remain in soils for only a few days. During this period, decreased nitrification and $N_2O$ emissions are observed, but these few days of inhibitory activity were not sufficient to produce overall significant effects.

It is clear from the results of this experiment and the results of Chapter 4 that the rate of aucubin applied, which corresponds to approximately 10% of the highest calculated potential aucubin excretion rate from 4.4.4, is not high enough to produce a significant overall effect within the short period of active inhibition. Therefore, an experiment that evaluates the effect of increasing the aucubin application rate on urine patch nitrification dynamics is warranted. This experiment is performed and discussed in Chapter 6.

# Chapter 6
# Urine patch $N_2O$ emissions under varying rates of aucubin application

## 6.1 Introduction

Urine N deposition onto grazed pasture soil is a significant source of nitrous oxide ($N_2O$), a greenhouse gas (GHG) with 298 times the global warming potential of carbon dioxide ($CO_2$) (Forster et al. 2007). In New Zealand, agricultural soils are the dominant source of national $N_2O$ emissions, primarily driven by urine deposition onto soils (Oenema et al. 1997; Oenema et al. 2005; Ministry for the Environment 2016). The soil processes which transform urine N, which consists primarily of urea, into $N_2O$ are well known (Haynes & Williams 1993; Selbie et al. 2015).

Urea is hydrolysed to ammonium ($NH_4^+$), which is then oxidised to form nitrate ($NO_3^-$) (Equation 2.1, Equation 2.2). The oxidation of $NH_4^+$ to $NO_3^-$ is known as nitrification, and $N_2O$ can be produced from intermediates formed during this process, or it may be produced during the reduction of $NO_3^-$ to $N_2O$ via denitrification (Mathieu et al. 2006; Carter 2007). Inhibiting nitrification in soils to reduce $N_2O$ loss has recently been a key mitigation focus and has shown great potential for reducing urine patch $N_2O$ loss (Di & Cameron 2002a; Moir et al. 2007; Cameron et al. 2014; Ruser & Schulz 2015; Di & Cameron 2016). Inhibiting nitrification also reduces the amount of $NO_3^-$ available for loss via leaching, which is another critical environmental issue in pasture ecosystems (Di & Cameron 2016).

Several inhibitors that can significantly reduce nitrification in the urine patch, and thus $N_2O$ emissions from pasture soils, have been identified (Di & Cameron 2002a; Cameron et al. 2014; de Klein et al. 2014a; Ledgard et al. 2014; Ruser & Schulz 2015; Cai et al. 2017). However, there are several issues with the implementation of these inhibitors that include: specific targeting of the urine patch with the inhibitor, translocation of the inhibitory chemicals into agricultural food products such as milk, chemical costs, and additional labour inputs for farmers (Di & Cameron 2016). Therefore, there remains an urgent need to identify novel, easily implemented nitrification inhibitors that can be rapidly implemented in New Zealand pasture systems.

Previous studies have identified that aucubin, a plant secondary metabolite (PSM) in the pasture herb plantain, is a potential nitrification inhibitor (Davini et al. 1986; Bartholomaeus & Ahokas 1995; Dietz et al. 2013; Gardiner et al. 2016). Aucubin is typically found in plantain leaves at concentrations of ~1% DM, but can reach concentrations up to 7%, depending on leaf age, season, or previous herbivory (Bowers & Puttick 1988; Adler et al. 1995; Darrow & Bowers 1999; Marak et al. 2002; Tamura & Nishibe 2002; Fuchs & Bowers 2004; Wurst et al. 2010). Livestock that graze pasture containing plantain naturally ingest aucubin as a part of their diet. As a result, they may potentially

excrete aucubin in their urine, thereby directly applying a nitrification inhibitor to the urine patch (Gardiner et al. 2016). This application technique avoids the implementation issues associated with other chemical nitrification inhibitors noted above. However, aucubin has not been evaluated as a nitrification inhibitor under urine patch conditions. Chapters 4 and 5 explored and evaluated the inhibitory capacity of aucubin in urine-affected soils, but, the potential aucubin excretion rate from livestock grazing plantain, and therefore the potential aucubin loading rate in the urine patch, remains unknown.

Aucubin is an iridoid glycoside with an *O*-linked glucose molecule at C-1 (Figure 2.5A). β-glucosidase can hydrolyse the glucose to form the aglycone, known as aucubigenin, which is the active anti-microbial product of aucubin (Davini et al. 1986). One study concluded that rumen microbes degraded aucubin without being negatively affected by the inhibitory activity of aucubigenin (Navarrete et al. 2016). Therefore, it is perhaps also possible that 100% of the aucubin consumed is excreted in urine, but it is likely excreted as its aglycone, aucubigenin. However, for the experiments performed in this PhD thesis, aucubin, rather than aucubigenin, was added to urine, due to the high cost and lack of availability of aucubigenin.

Previous research on plantain, focussing on the effects of plantain in the forage diet of ruminants, has included plantain in the diet at percentages that range from 15-100% (Edwards et al. 2015; Box et al. 2016; Judson & Edwards 2016; Cheng et al. 2017; Nkomboni 2017). It was calculated in Section 4.4.4 that the potential aucubin excretion rate within a urine patch area, for a 100% plantain diet, assuming 100% excretion of aucubin, could be equivalent to 487 kg $ha^{-1}$, based on assumptions of urinary volume and urinary aucubin concentrations (Darrow & Bowers 1999; Wurst et al. 2010; Moir et al. 2011; Selbie et al. 2015; Box et al. 2016). However, the actual percentage of consumed aucubin that is excreted in urine remains unknown.

The rates of aucubin used in Chapters 4 and 5 (47-96 kg $ha^{-1}$) were based on the prior work of Dietz et al. (2013), but that study was not performed under urine patch conditions. Thus it remains possible that higher rates of aucubin application would further reduce nitrification in the urine patch and subsequent $N_2O$ emissions. It is likely that increasing rates of aucubin application in the urine patch would lead to increased nitrification inhibition, because Dietz et al. (2013) found that increasing rates of plantain leaf extract, which contains aucubin, led to increasing degrees of nitrification inhibition. Therefore, it is necessary to evaluate the effects of increased rates of aucubin application on urine patch N dynamics to determine whether there is a critical threshold of aucubin concentration that leads to significant reductions in urine patch nitrification.

The aim of this experiment was to determine the effect of various aucubin application rates on nitrification and $N_2O$ emissions following urine application to soil. Three aucubin application rates were chosen:

1. 47 kg $ha^{-1}$, the rate used in both field trials, based on Dietz et al. (2013)
2. 243 kg $ha^{-1}$, half the highest calculated potential aucubin excretion rate from Section 4.4.4
3. 487 kg $ha^{-1}$, the highest calculated potential aucubin excretion rate from Section 4.4.4

It was hypothesised that applying aucubin in urine would reduce both $NO_3^-$ accumulation in soils and soil $N_2O$ emissions, with increased concentrations of aucubin application leading to increased reduction in $NO_3^-$ accumulation and $N_2O$ emissions.

## 6.2 Methods

### 6.2.1 Laboratory trial set-up and treatments

A Paparua sandy loam soil (Typic Immature Pallic (NZ)/Udic Ustochrept (USDA)), under a mixed PR-WC pasture, was collected (0-10 cm depth) from the Lincoln University Research Dairy Farm (43°38'25.36"S, 172°27'25.05"E) on July 5, 2017. The soil was sieved to 4 mm and stored at 4°C until use. The soil contained 3.9% organic matter, 2.3% total C, and 0.24% total N, with a C/N ratio of 9.5 (Hill Laboratories). On July 20, 25.4 g fresh weight soil (20 g dry weight equivalent) was placed in 30 mL plastic vials (ThermoFisher Scientific) and incubated in the laboratory at 18-20°C for 11 days in a complete randomised design. Any germinating seedlings were removed and soil moisture was maintained at approximately 27% gravimetric soil water content (equivalent to ~44% WFPS, assuming 1 g $cm^{-3}$ BD) for the duration of the incubation and experimental period, by additions of deionised water, as needed.

Urine was collected from dairy cows grazing PR-WC pasture on the Lincoln University Commercial Dairy Farm (43°38'39.8"S, 172°26'33.7"E) on July 3 and stored at -4°C before use. Urine N (see 4.2.1.1) and urea content (Gribbles Veterinary Clinic, Christchurch, New Zealand) were analysed before and after freezing to ensure there were no freezing effects on urine N content or composition. The urine contained 6.63 g N $L^{-1}$. Treatments for this trial included a nil-N control, urine control, and three urine + aucubin treatments: control (4.8 mL deionised water), urine only (4.8 mL urine), Urine + A1 (4.8 mL urine + 2.2 mg aucubin), Urine + A2 (4.8 mL urine + 11.0 mg aucubin), and Urine + A3 (4.8 mL urine + 22.0 mg aucubin).

**Table 6.1. Urine N and aucubin application rates for the five treatments used in this laboratory trial.**

| Treatment | Urine application rate (kg $ha^{-1}$) | Aucubin application rate (kg $ha^{-1}$) |
|---|---|---|
| **Control** | 0 | 0 |
| **Urine** | 700 | 0 |
| **Urine + A1** | 700 | 47 |
| **Urine + A2** | 700 | 243 |
| **Urine + A3** | 700 | 487 |

The treatments were applied as a solution, with additions of 70.6, 364, and 728 mg aucubin to 160 mL urine for the Urine + A1, Urine + A2 and Urine + A3 treatments, respectively. All urine treatments received 700 µg N $g^{-1}$ dry soil, and aucubin rates were based on rates used in Dietz et al. (2013) and Gardiner et al. (2017), resulting in additions of 0.11, 0.55, and 1.1 mg aucubin $g^{-1}$ dry soil in the 47, 243, and 487 kg $ha^{-1}$ aucubin treatments, respectively. Aucubin was purchased from Nanjing Leading Chemical Co. (Nanjing, China).

Treatments were applied to 5 sets of vials, each with 4 reps per treatment. These 5 sets provided for successive destructive analysis over 5 time points. Treatments were applied on July 31 (Day 0) and vials were placed into a randomised block design and incubated in the laboratory at 18-20°C for the remainder of the experiment. One set of vials was destructively analysed for inorganic N content and soil moisture on Days 1, 7, 17, 28, and 37 (Table 6.2).

**Table 6.2 The sets of vials available for $N_2O$, $CO_2$, and surface pH analysis (left), and the dates of destructive soil analysis on each set (right). A random remaining set was chosen for sampling of $N_2O$, $CO_2$, and surface pH, similar to 4.2.1.**

| Days | Sets remaining for $N_2O$, $CO_2$, and pH analysis | Day | Set destroyed for soil analysis |
|---|---|---|---|
| 0-1 | 1, 2, 3, 4, 5 | 1 | 1 |
| 2-7 | 2, 3, 4, 5 | 7 | 2 |
| 8-17 | 3, 4, 5 | 17 | 3 |
| 18-28 | 4, 5 | 28 | 4 |
| 29-37 | 5 | 37 | 5 |

For each sampling day of $N_2O$ flux, $CO_2$ flux, and surface pH measurements one of the remaining sets of vials was randomly chosen (Table 6.2), so that variability between the sets was accounted for, as per the methods of 4.2.1.

### 6.2.2 Gas flux measurements: $N_2O$ and $CO_2$

Nitrous oxide emissions were measured every 1-3 days by placing each remaining vial (see Table 6.2) into a sealable glass jar, creating a 285 $cm^3$ headspace. The jar was sealed immediately after placing the vial inside (t0), and samples were collected using a 30 mL glass syringe fitted with a three-way stopcock at 0, 30, and 60 minutes (t0, t30, and t60).

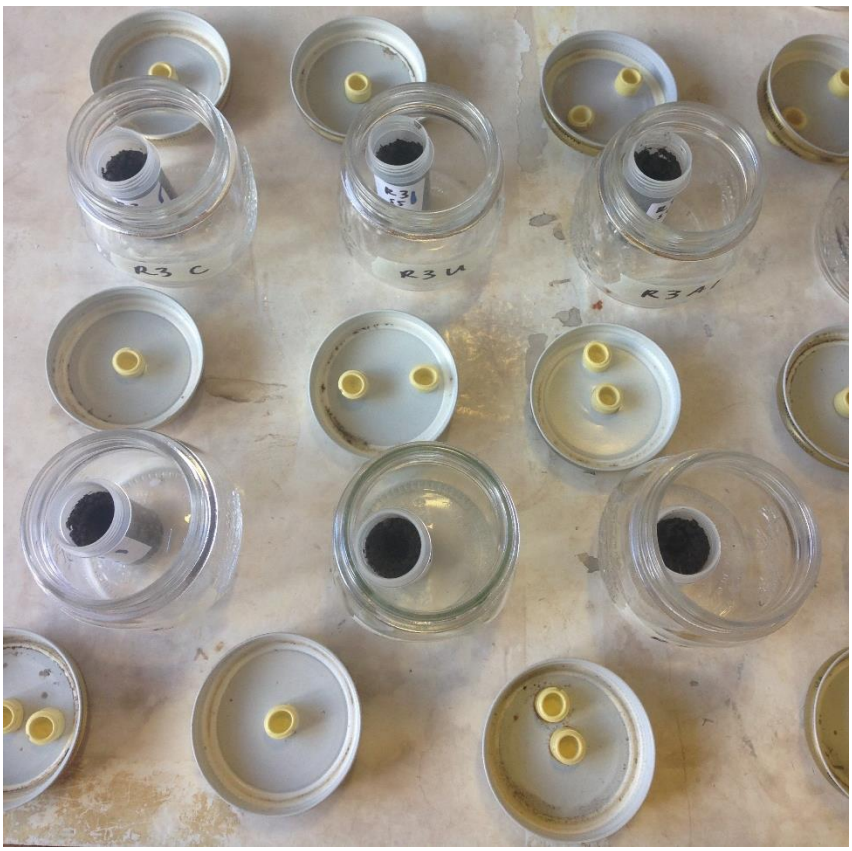



**Figure 6.1 The vials inside the open gas sampling jars after completing a sampling. Gas samples were taken with a 30 mL syringe through the rubber septa in the lid.**

Headspace samples (12 mL) were transferred to pre-evacuated 6 mL glass vials (Exetainer, Labco Ltd.) which were then analysed using an automated gas chromatograph system as previously described (3.2.2.3). A linear relationship was observed between the change in headspace $N_2O$ concentration versus time (average $r^2 = 0.91$), therefore the $N_2O$ in the chamber headspace was determined using linear regression for all samples. Fluxes of $N_2O$ were calculated using: the change in $N_2O$ concentration over time, the ideal gas law, air temperature, chamber headspace volume and surface area (4.2.1.3.1). Since $N_2O$ sampling was performed on a random set of vials each week, as explained in 6.2.1 and as performed in 4.2.1.2, cumulative emissions and EFs were not calculated for this experiment.

Carbon dioxide emissions were determined every 2-6 days by placing the vial inside a glass jar with a 100 $cm^3$ headspace (Figure 6.2). The glass jar was sealed with a large rubber stopper fitted with inlet and outlet tubes (Bev-A-Line tubing) connected to a LI-820 closed-path gas analyser (LI-COR). The closed path sampling system included a Balston 25 micron air filter and air flow was maintained through the system at approximately 1 L $min^{-1}$ using a calibrated flow meter.

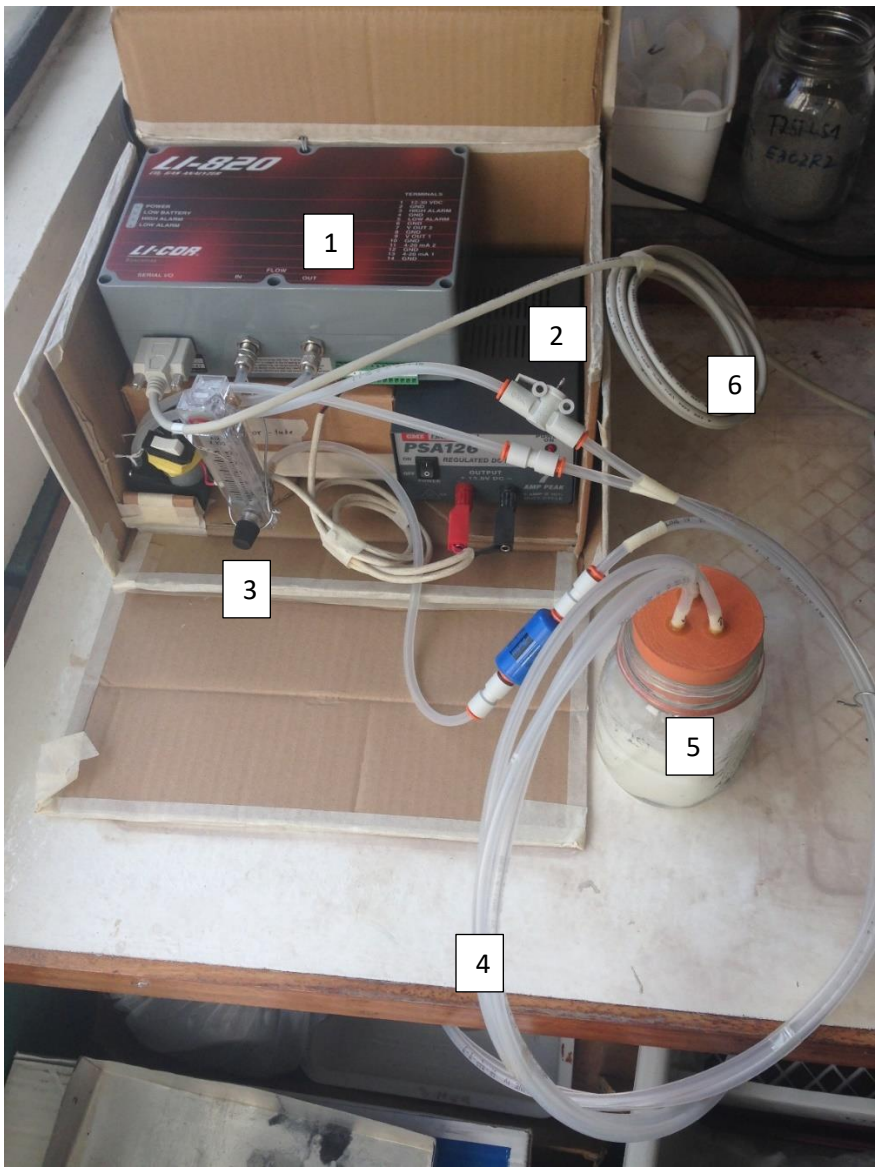



**Figure 6.2 The LI-COR sampling system, including the LI-COR analyser (1), battery (2), gas flow regulator (3), inlet and outlet tubes (4), sampling chamber (5), and connection to the computer for data collection (6).**

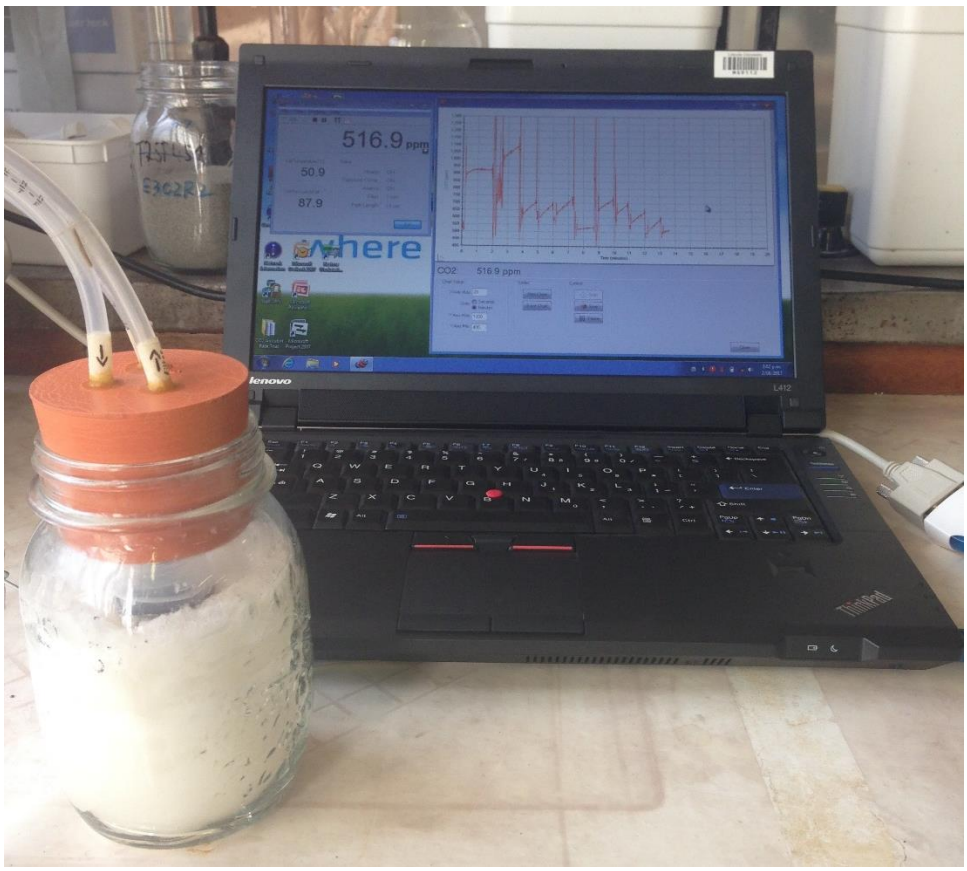



**Figure 6.3 A vial inside the $CO_2$ sampling chamber, with the real-time $CO_2$ μL $L^{-1}$ (ppm) and a graph of Δ μL $L^{-1}$ over time shown on the laptop screen.**

Concentration measurements were taken once a second for 1 minute (Figure 6.3). Between vials, the LI-COR system was flushed until background ambient concentrations of $CO_2$ (μL C $L^{-1}$) were reached. Fluxes of $CO_2$ were calculated using the linear increase in $CO_2$ concentration over time, with the average $r^2$ value for all samples was 0.79.

### 6.2.3 Soil inorganic N analysis

Soil inorganic N was measured by destructively sampling one complete set of vials on Days 1, 7, 17, 28, and 37. The extraction and analyses were performed according to the methods previously described in 3.2.1. Gravimetric soil moisture was determined for each vial at each destructive analysis according to the methods in 3.2.1. Surface pH was measured on Days 1, 4, 7, 11, 16, 22, 28, 32, and 36 according to the methods in 3.2.2.4.

### 6.2.4 Statistical analysis

Data were analysed using Genstat Version 18. Residual plots generated by the ANOVA analysis in Genstat for each variable were visually analysed to check that the data met the assumptions for the ANOVA analysis. The equal variance of data was determined using the fitted-value plot. The normality of residuals was checked using the normal plot and half-normal plot, and tested using the Shapiro-Wilk normality test. Statistical analysis was performed on data sets that met the assumptions of the ANOVA analysis. All data, except overall $N_2O$ and $CO_2$ flux data, had a normal distribution. The overall $N_2O$ and $CO_2$ data were log transformed before analysis. An ANOVA was performed on each measured variable with Treatment and Block as factors. Least significant differences of means (5% level) were used to determine significance using Fisher's unprotected least significant difference test.

## 6.3 Results

### 6.3.1 Soil $N_2O$ flux

Peak $N_2O$ fluxes occurred on Day 37 in all urine-amended treatments, reaching a maximum of 3700 µg $N_2O$-N $m^{-2}$ $h^{-1}$ in the Urine + A3 treatment. When assessing daily fluxes (Figure 6.4A), the Urine + A3 treatment had a higher $N_2O$ flux than the Urine treatment on Days 1, 2, and 32 and a lower $N_2O$ flux on Days 7 and 9 ($P<0.05$). The Urine + A1 treatment produced a higher $N_2O$ flux than the Urine treatment on Day 11 ($P<0.05$). On Days 3, 5, and 6, the control treatment produced higher $N_2O$ fluxes than two or more of the aucubin treatments (Day 3: Urine + A1 and Urine + A2; Day 5: all aucubin treatments; Day 6: Urine + A2 and Urine + A3) ($P<0.05$), however, the overall average $N_2O$ flux from the control treatment was lower than all urine-amended treatments ($P<0.05$).

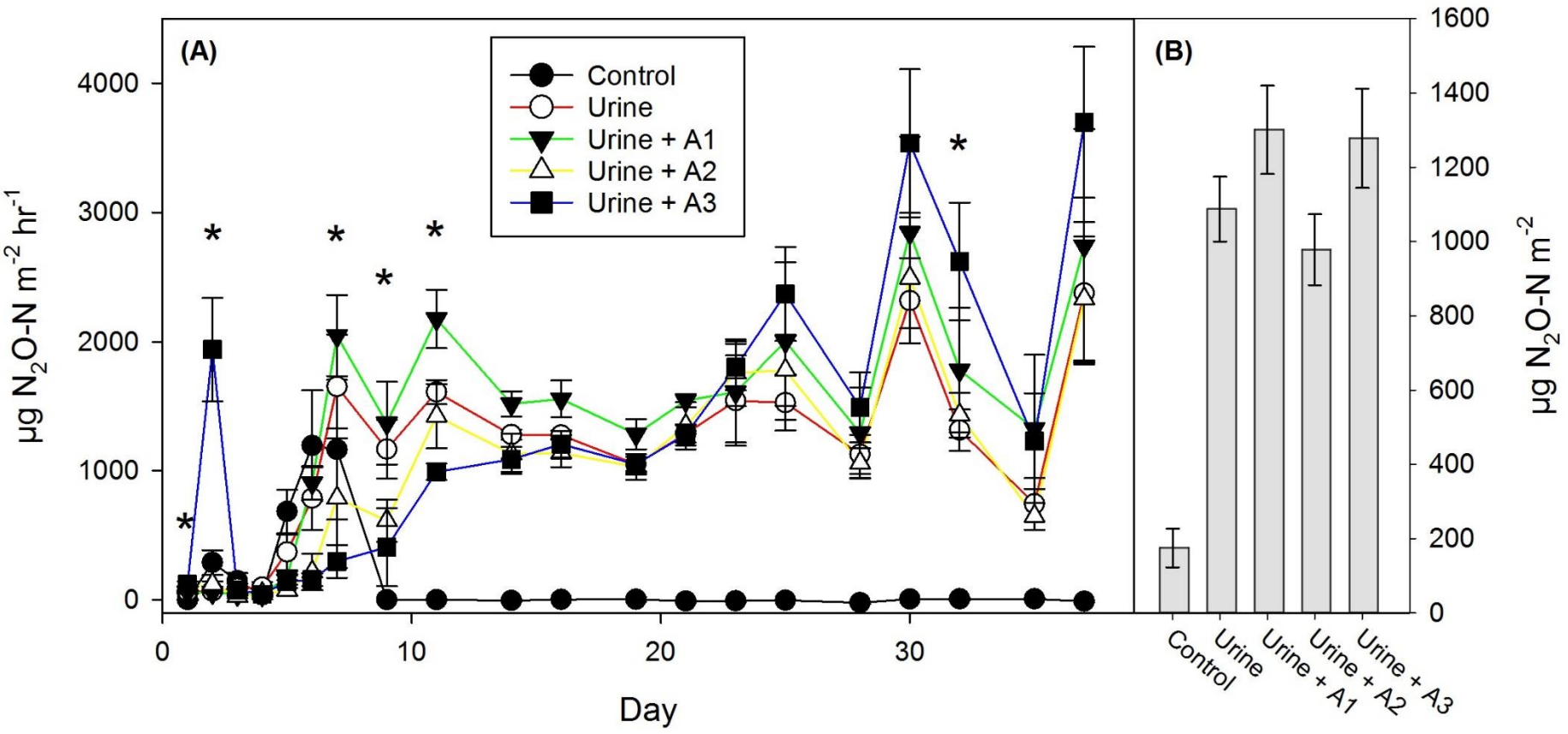



**Figure 6.4. Daily $N_2O$ flux from each of the five experimental treatments (A) and the average $N_2O$ flux over the 37 day experiment for each treatment (B). Data points represent means (A, n=4; B, n=98), with error bars (SEM). Stars indicate days when one or more aucubin treatments were significantly different than the Urine treatment ($P<0.05$).**

Over the study period, average $N_2O$ fluxes were 170, 1090, 1300, 980, and 1280 µg $N_2O$-N $m^{-2}$ $h^{-1}$ in the Control, Urine, Urine + A1, Urine + A2, and Urine + A3 treatments, respectively (Figure 6.4B). The overall average fluxes were not significantly different between urine-amended treatments. However, the average flux from Days 5-11 was significantly lower in the Urine + A2 and Urine + A3 treatments than in the Urine treatment ($P<0.05$; Figure 6.5).

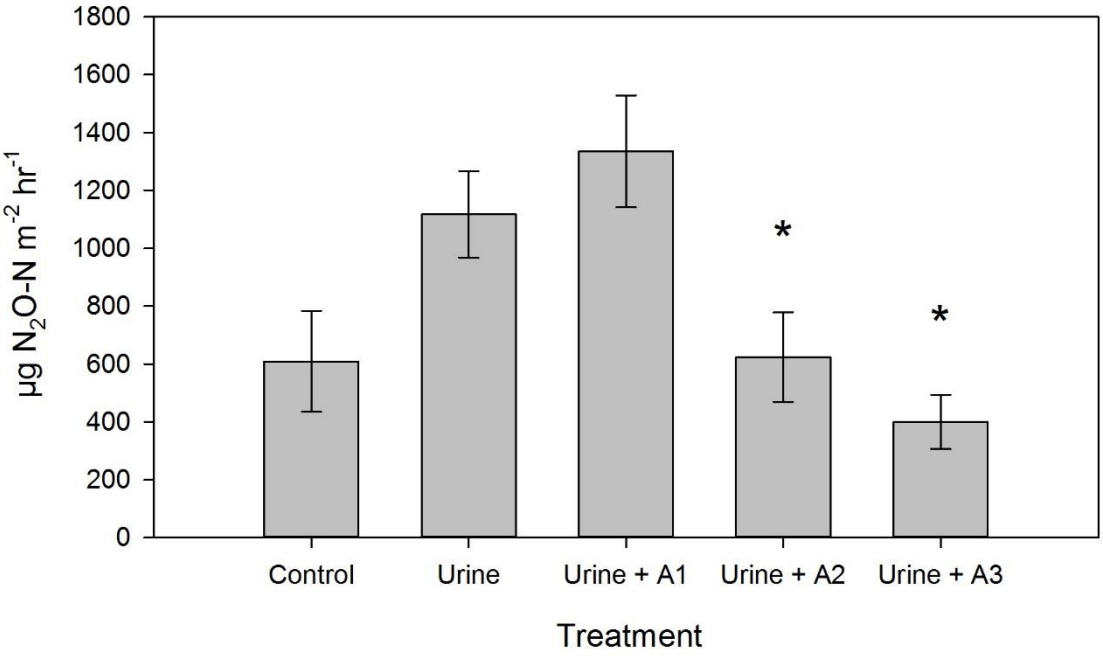



**Figure 6.5 The average $N_2O$ flux from Days 5-11 for each treatment (n=20). Error bars represent SEM and stars indicate aucubin treatments that were significantly different than the Urine treatment ($P<0.05$).**

### 6.3.2 Soil inorganic N

Soil $NH_4^+$ concentrations peaked on Day 1 in all urine-amended treatments (Figure 6.6). Over all sampling days, soil $NH_4^+$ concentrations averaged 2.08 µg $NH_4^+$-N $g^{-1}$ soil in the control treatment, and 758, 769, 752, and 772 µg $NH_4^+$-N $g^{-1}$ soil in the Urine, Urine + A1, Urine + A2, and Urine + A3 treatments, respectively.

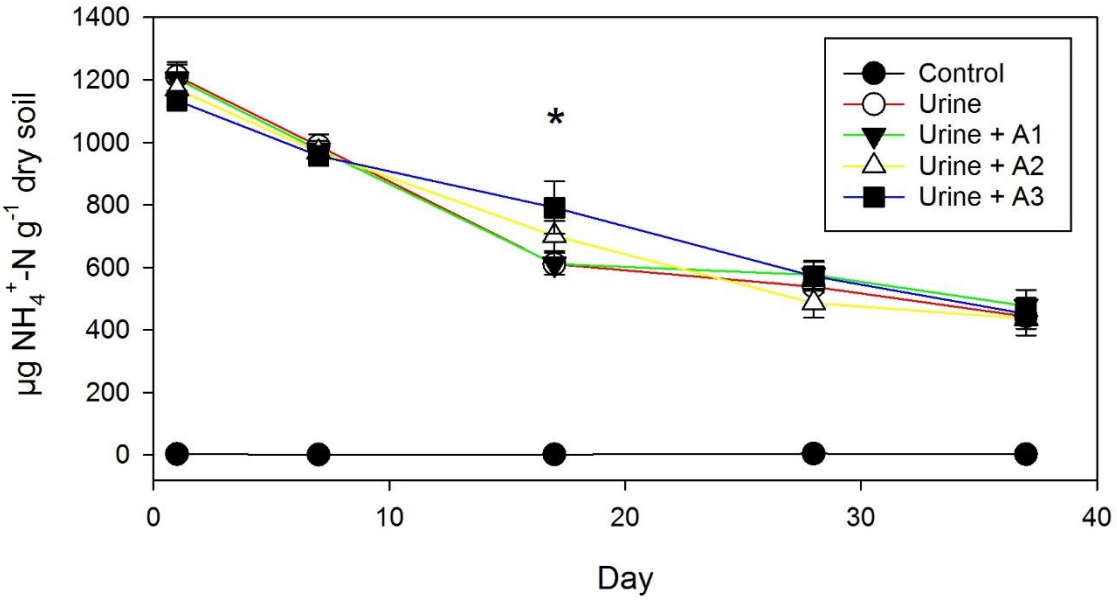



**Figure 6.6. Soil $NH_4^+$ concentrations in the five treatments over the 37 day experimental period. Data points represent means (n=4) with error bars (SEM). Stars indicate days when one or more aucubin treatments were significantly (P<0.05) different than the Urine treatment.**

On Day 17, the Urine + A3 treatment contained higher soil $NH_4^+$ concentrations than the Urine treatment (P<0.05; Figure 6.6). There were no significant differences in soil $NH_4^+$ concentrations between urine-amended treatments on any other days, or when averaged over the entire experimental period. The control treatment contained lower soil $NH_4^+$ concentrations than all urine-amended treatments on all days (P<0.05).

Soil $NO_3^-$ concentrations averaged 45.4, 242, 251, 213, and 192 μg $NO_3^-$-N $g^{-1}$ soil in the control, Urine, Urine + A1, Urine + A2, and Urine + A3 treatments, respectively. Peak soil $NO_3^-$ concentrations occurred on Day 17 in the Urine treatment, and on Day 28 in the Urine + A1, Urine + A2, and Urine + A3 treatments.

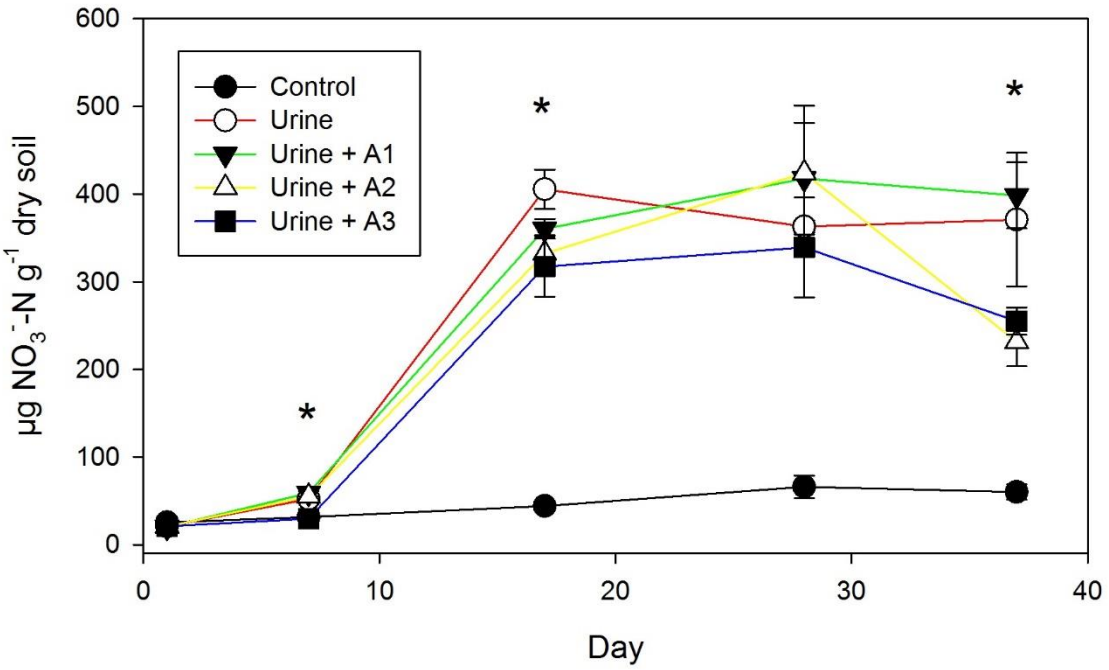



**Figure 6.7. Soil $NO_3^-$ concentrations over the 37 day experimental period. Data points represent means (n=4) with error bars (SEM). Stars indicate days when one or more aucubin treatments were significantly different (P< 0.05) than the Urine treatment.**

Soil $NO_3^-$ concentrations were lower in the Urine + A3 treatment than the Urine treatment on Days 7, 17, and 37 ($P<0.05$; Figure 6.7). The Urine + A2 treatment also contained lower soil $NO_3^-$ concentrations than the Urine treatment on Day 17 and 37 ($P<0.05$). However, when averaged over the entire experimental period, there were no significant differences in soil $NO_3^-$ concentrations between urine-amended treatments. The control treatment contained lower soil $NO_3^-$ concentrations than all urine-amended treatments on all days ($P<0.05$).

### 6.3.3 Soil $CO_2$ flux

Averaged over all sampling days, $CO_2$ fluxes were 65.8, 200, 202, 186, and 185 mg $CO_2$-C $m^{-2}$ $h^{-1}$ in the control, Urine, Urine + A1, Urine + A2, and Urine + A3 treatments, respectively. All aucubin treatments had significantly lower average $CO_2$ fluxes than the Urine treatment on Day 4 ($P<0.05$; Figure 6.8).

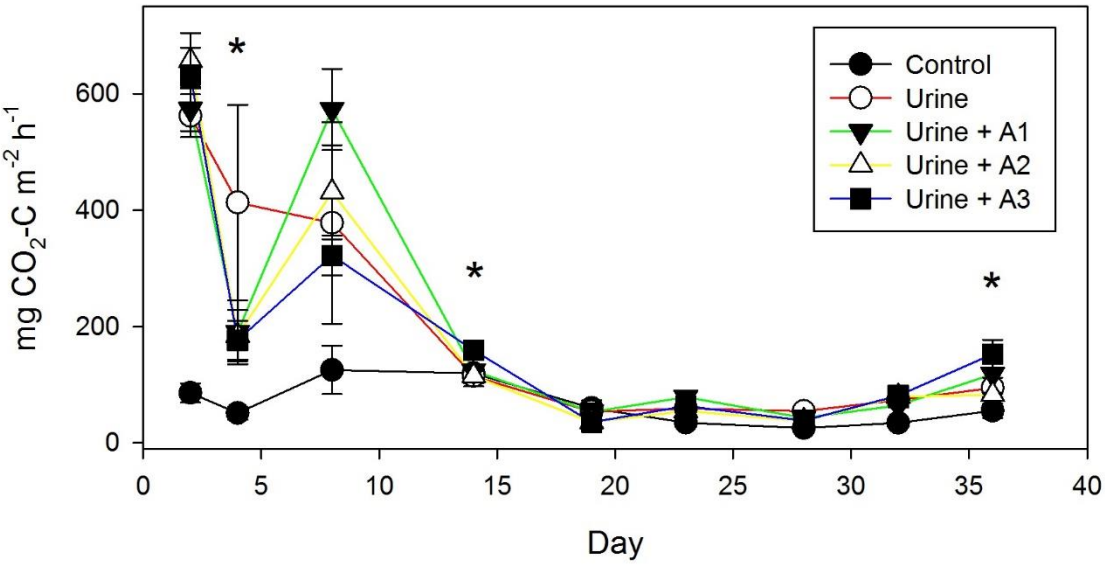



**Figure 6.8. Soil $CO_2$-C fluxes over the 37 day experimental period. Data points are means (n=4) with error bars (SEM). Stars indicate days when one or more aucubin treatments were significantly different (P<0.05) than the Urine treatment.**

Fluxes of $CO_2$ were higher in the Urine + A3 treatment when compared to the Urine treatment on Days 14 and 36 (P<0.05; Figure 6.8). Differences in $CO_2$ flux between aucubin treatments occurred on Day 23, when the Urine + A1 flux was significantly higher than the Urine + A2 flux, and on Day 36, when the Urine + A3 flux was significantly higher than the Urine + A2 flux (P<0.05). One or more urine-amended treatments were statistically similar to the Control on all days except Day 2 and 32. Overall, the control treatment averaged lower $CO_2$ fluxes than the urine-amended treatments (P<0.05), and all urine-amended treatments were statistically similar.

### 6.3.4 Soil surface pH

Overall, soil surface pH peaked on Day 1, with an average of 7.13 in all urine-amended treatments (Figure 6.9). The surface pH in the Urine + A3 treatment was higher than the Urine treatment on Days 11 and 16 (P<0.05). The surface pH in the Urine + A2 treatment was significantly higher than the Urine treatment on Day 16 (P<0.05), and the Urine + A1 treatment was not significantly different than the Urine treatment on any day.

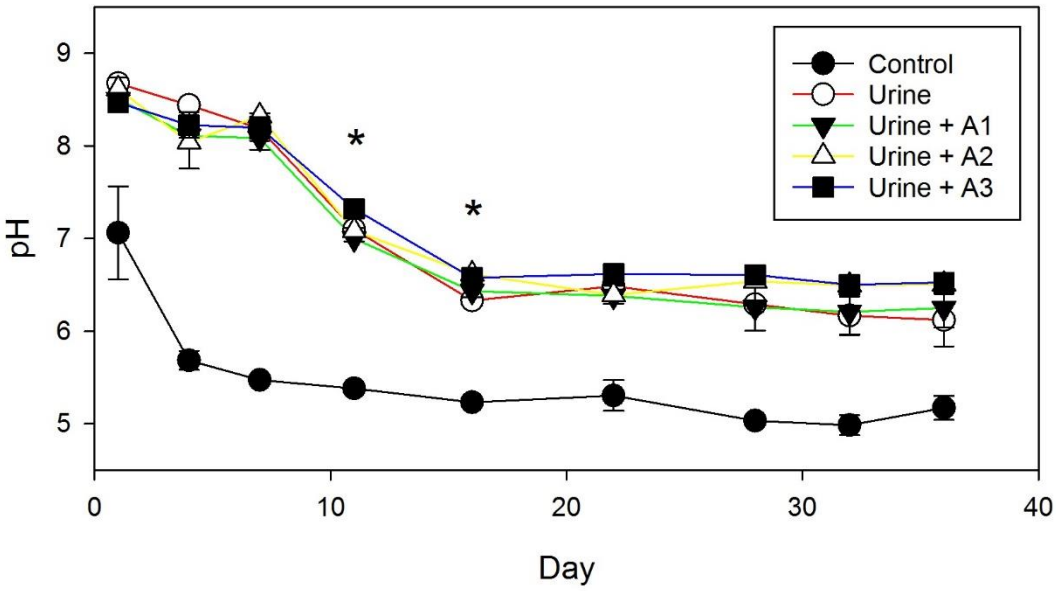



**Figure 6.9. Soil surface pH over the 37 day laboratory experiment. Data points are means (n=4) with error bars (SEM). Stars indicate days when one or more aucubin treatments were significantly different (P<0.05) than the Urine treatment.**

When averaged over the whole experiment, surface pH was not significantly different between urine-amended treatments. Surface pH was lower in the control treatment than all urine-amended treatments on all days (P<0.05).

## 6.4 Discussion

### 6.4.1 Nitrous oxide emissions

Consistent with our hypothesis, $N_2O$ emissions were reduced in the Urine + A2 and Urine + A3 treatments from Days 5-11, with an increasing rate of aucubin application leading to lower emissions. However, this period of inhibition was not sufficient to produce an overall statistically significant reduction in soil $N_2O$ emissions when aucubin, at any tested rate, was added to urine.

The expected decrease in $N_2O$ emissions may have been offset due to the increased C applied in the aucubin treatments, which may have stimulated heterotrophic microbial activity, particularly denitrifiers, and thus $N_2O$ production. The rate of aucubin applied within the three aucubin treatments resulted in an additional 5.7, 28.6, and 57.2 µg C $g^{-1}$ dry soil being added to the Urine + A1, Urine + A2, and Urine + A3 treatments, respectively. This C addition may have stimulated microbial activity because aucubin contains a glucose ring (Figure 2.5C) which is a readily available C source for microbial growth (Behera & Wagner 1974). Hence, it might be argued that the peak in the $N_2O$ flux on Day 2 from the Urine + A3 treatment occurred due to the increase in C added within this

treatment. However, such an argument is not supported by the $CO_2$ data, since there were no differences in the $CO_2$ fluxes on Day 2, when the $N_2O$ peak occurred, indicating that there was no difference in microbial respiration at this time.

Apart from Days 11 and 16, there were no differences in pH between the Urine and the three aucubin-amended treatments. This indicates that, regardless of treatment, processes affecting the soil pH (ammonia volatilisation, nitrification, and denitrification (Haynes & Williams 1992)) had a similar net effect.

Consumption of $N_2O$ is also favoured by elevated pH because the activity of $N_2O$ reductase, which converts $N_2O$ to $N_2$, is disrupted under low pH (Bergaust et al. 2010; Liu et al. 2010; Liu et al. 2014; Brenzinger et al. 2015; Samad et al. 2016). However, the lack of any consistent treatment effects, with increasing aucubin rate, on soil pH means that consumption of $N_2O$ is unlikely to have influenced $N_2O$ fluxes via changes in the $N_2O$:$N_2$ ratio. The amount of labile C available for denitrifiers may also favour $N_2O$ consumption to $N_2$, but despite the additional C supplied in the aucubin, no decline in $N_2O$ flux was observed. This may be a consequence of the soil moisture content favouring nitrification rather than denitrification (Bateman & Baggs 2005). Thus, differences in $N_2O$ emissions between treatments are not likely due to differences in $N_2O$:$N_2$ ratios.

The rate of urea hydrolysis, a rapid process that is typically complete within 48 hours after urine deposition (Williams & Haynes 1994), is another factor which can affect the rate of formation of the $NH_4^+$ pool available for subsequent nitrification, and any ensuing $N_2O$ production. Urea hydrolysis is facilitated by the urease enzyme and commercial urease inhibitors target the activity of this enzyme as a method of slowing $NH_4^+$ availability after urine deposition, to increase the potential plant uptake of urine N and thereby reduce N available for $N_2O$ production (Zaman et al. 2009). Since there was no observed difference in the timing of the soil $NH_4^+$ concentration or soil pH dynamics as a consequence of treatment, the effect of aucubin on urease can be assumed to be negligible, similar to the results of Chapters 4 and 5.

Lower $N_2O$ fluxes in the first 4 days, with the exception of the peak in the Urine + A3 treatment on Day 2, may be due to $NH_3$ toxicity, which is known to inhibit nitrification (Anthonisen et al. 1976; van Cleemput & Samater 1995; Venterea et al. 2015). A similar trend occurred in the laboratory trial in Chapter 4, when a high concentration of $NH_4^+$ and $NH_3$ inhibited microbial activity and $N_2O$ emissions, as explained in 4.4.1. The vials in this laboratory trial were incubated on the laboratory bench, rather than in an incubator (as done in 4.4.1), to increase air flow around the vials, with the aim of reducing $NH_3$ build-up and resulting $NH_3$ toxicity. Additionally, the control treatment showed an elevated pH on Day 1 and increased $N_2O$ flux on Days 5-7, recording similar $N_2O$ flux to almost all urine-amended treatments. This is likely to have been due to $NH_3$, alkaline in nature, being deposited onto soil in the

control vials, since they were randomised within the urine-amended treatments, indicating $NH_3$ was present. Volatilisation of $NH_3$ typically peaks 1-2 days after urine deposition (Selbie et al. 2015), and 20-60% of volatised $NH_3$ can be redeposited within 2 m of the deposition site (2.3.3.1; Ross & Jarvis 2001).

Despite being a laboratory experiment, the $N_2O$ fluxes measured during this experiment are within the previously reported range of $N_2O$ fluxes for pasture soils in New Zealand (de Klein et al. 2003). These fluxes are much higher than those recorded in the laboratory trial in Chapter 4, likely because the degree of $NH_3$ inhibition was much lower, and the resulting nitrification rates and availability of $NO_3^-$ were much higher. Additionally, fluxes measured in this trial are higher than those measured in both Objective 1 and 2 of the field trial in Chapter 4. The increased fluxes in this laboratory trial are likely due to the fact that applied N is confined to the experimental vial, and cannot be removed via plant N uptake or leaching, therefore a much higher proportion of applied N was available as substrate for $N_2O$ emission pathways.

### 6.4.2 Nitrification inhibition

Despite the inconclusive effects of elevated aucubin on overall average $N_2O$ fluxes, it appears that nitrification inhibition, caused by the antimicrobial capacity of aucubin, led to the significantly lower $N_2O$ flux rates from Days 5-11 measured in the Urine + A2 and Urine and Urine + A3 treatments, when compared to the Urine treatment. This is supported by the results of other measured variables, such as soil inorganic N concentrations and soil surface pH, which all indicate that nitrification inhibition was occurring at this time.

Increased $NH_4^+$ concentrations and decreased $NO_3^-$ concentrations, or a delayed increase in $NO_3^-$ concentrations, have been used as indicators of nitrification inhibition (Baral et al. 2014; Cameron et al. 2014; de Klein et al. 2014a; Di & Cameron 2016). This was observed in our experiment, where soil $NH_4^+$ concentrations were significantly higher in the Urine + A3 treatment than in the Urine treatment on Day 17, while soil $NO_3^-$ concentrations were significantly lower in the Urine + A3 treatment on Days 7 and 17. Furthermore, peak soil $NO_3^-$ concentrations occurred later in all the aucubin treatments than in the Urine treatment.

Additionally, the elevated soil surface pH in the Urine + A3 treatment on Day 11 and in both the Urine + A2 and Urine + A3 treatment on Day 16, relative to the control, indicated that nitrification, the process which reduces soil pH from its urine-induced alkaline state (see 2.3.3.4), was not occurring as rapidly in these treatments at this time. It is important to note that pH is a net value, and that other processes which increase soil pH, such as denitrification, which uses $H^+$ to form $H_2O$ in

the reduction of $NO_2^-$ (Wrage et al. 2001), may have also contributed to the increased pH during this time. However, it is not likely that denitrification contributed significantly to the measured soil pH because the soil conditions, particularly soil WFPS, were not ideal for high rates of denitrification (Firestone 1982).

Therefore, the combination of soil inorganic N concentrations and soil surface pH from Days 5-17 indicate that aucubin inhibited nitrification, and that this inhibition led to the significantly lower average $N_2O$ flux in the Urine + A2 and Urine + A3 treatments, compared to the Urine treatment from Days 5-11. However, this effect was not prolonged enough to produce a significant reduction in overall average $N_2O$ flux during the 37 day experiment, potentially due to the decomposition of aucubin in soil.

The short inhibitory period measured in this experiment is similar to the inhibitory period measured in Objective 1 of Chapter 4 and in Chapter 5. As discussed in 5.4.4, it is likely that aucubin is degraded within the first 4 days of the experiment to form its active aglycone, aucubigenin, which then seems to inhibit nitrification for the following few days. As aucubigenin is typically in equilibrium with its highly reactive dialdehyde form (Ghisalberti 1998), it reacts via cross-linking to form a variety of compounds (Migneault et al. 2004). From the results of this experiment, and results of Chapters 4 and 5, it appears that aucubigenin, or its derivatives, has an active inhibitory period of approximately 4-10 days in the urine patch.

### 6.4.3 Fate of aucubin in the urine patch

The short period of inhibitory activity by aucubin, as determined when considering all the $N_2O$, $CO_2$, soil inorganic N, and soil pH data, is consistent with the results found in Chapter 4, where aucubin was found to rapidly degrade in soil. In that laboratory experiment, it was found that aucubin completely degraded in soil within approximately 4 days (Table 4.1). However, in the following field trial, it was found that aucubin degraded within 24 hours of application (4.3.2.2). As explained in 4.4.2, aucubin can be degraded by β-glucosidase, an enzyme found in soils, within 4 hours (Busto & Perez-Mateos 1995; Busto & Perez-Mateos 2000; Kim et al. 2000). The products of aucubin degradation can be further transformed, and the previous soil extractions performed in Chapter 4 to measure the residence time of aucubin in the urine patch did not account for the residence times of the degradation products of aucubin. Therefore, as proposed in 4.4.2, soil samples were taken from this experiment for further analysis of the degradation products of aucubin, particularly aucubigenin, using liquid chromatography-mass spectrometry. However, due to budgetary restraints, these samples were not able to be analysed. Therefore, data on aucubin residence times, or further details on the degradation products of aucubin, need to be gathered.

### 6.4.4 Differences in soil $NO_3^-$ content and implications for further $N_2O$ emissions

As this experiment was performed as a laboratory soil incubation, with no plants present and no drainage from sampling vials, there was no soil N removal via plant N uptake or leaching. However, soil $NO_3^-$ concentrations were significantly lower in the Urine + A2 and Urine + A3 treatments than the Urine treatment on Day 37, while there was no significant difference in $NO_3^-$ concentrations between these treatments on the previous sampling day. It is possible that this $NO_3^-$ was removed via denitrification, which is supported by the heightened $N_2O$ flux in the Urine + A3 treatment from Day 31-37, but the difference in $N_2O$ flux was not significant and the soil moisture conditions were likely not wet enough for significant rates of complete denitrification to $N_2$ (Balaine et al. 2016). It is possible that the increased C content of the aucubin treatments stimulated $N_2$, rather than $N_2O$, production, as this is a known influencing factor of the $N_2O$:$N_2$ ratio (Firestone & Davidson 1989). The only other pathway for $NO_3^-$ removal is immobilisation, which could be increased due to the increased C concentrations added in these treatments stimulating microbial activity, as explained in 6.4.1.

This significantly lower $NO_3^-$ concentration in the Urine + A2 and Urine + A3 treatments at Day 37 suggests that, if the experiment were to have continued, future $N_2O$ flux in these treatments would likely be lower than in the Urine treatment, since $NO_3^-$ availability is a primary driver for denitrification and subsequent $N_2O$ emissions (Firestone & Davidson 1989). Therefore, it is possible that significant differences in $N_2O$ flux would have occurred after Day 37 if the sampling period had continued.

## 6.5 Conclusions

Adding aucubin, at rates equal to the calculated highest potential aucubin excretion rate, to urine applied to soils did not reduce overall soil $N_2O$ emissions over a 37 day period following urine application, contrary to our hypothesis. However, differences in daily $N_2O$ fluxes, soil $NH_4^+$ and $NO_3^-$ concentrations, and soil surface pH indicate that aucubin inhibited nitrification around the period of Days 7-17 in the Urine + A2 and Urine + A3 treatments. In these treatments aucubin was applied at the calculated highest potential aucubin excretion rate (A3) or half the calculated highest potential aucubin excretion rate (A2). Therefore, it is possible that livestock consumption of plantain, and the subsequent excretion of aucubin into the urine patch, could reduce urine patch $N_2O$ emissions due to the inhibitory capacity of aucubin, but the percentage of plantain in the diet and the percentage of aucubin excreted in urine would need to be at the highest possible levels. The urinary excretion rate from livestock grazing various concentrations of plantain and the factors that affect the urinary excretion rate of aucubin, remain unknown. Future studies must quantify the rate of aucubin and/or

its derivatives excreted in urine to determine whether aucubin can be excreted at a rate high enough to alter urine patch N dynamics.

It is possible that the inhibitory effects of aucubin do not last as long in soil, due to its degradation by enzymes and/or microbes, but the residence time of aucubin and its degradation products remains unknown. The measurement of these compounds in soil, and the determination of factors affecting their degradation, is a key next step in research on aucubin as a nitrification inhibitor. Ideally, this could be examined using $^{13}C$ labelled aucubin to enable the fate of the aucubin and its retention time to be evaluated.

# Chapter 7
# General discussion and conclusions

## 7.1 General overview

Urine patches in grazed pastures are a major source of $N_2O$, a potent greenhouse gas (Ministry for the Environment 2015a). This PhD thesis evaluated two novel methods for reducing urine patch $N_2O$ emissions via dietary manipulation of urine composition:

- Altering urine N composition to increase the proportion of urine N excreted as non-urea urine nitrogen compounds (NUNCs). These NUNCs may be less labile forms of N, capable of stimulating plant N uptake, or forms of N that degrade to compounds which inhibit nitrification, a key step in soil $N_2O$ production.

- Adding plant species into ruminant diets that contain an active PSM which may inhibit nitrification. Hypothetically, the PSM would be ingested and excreted in the urine, thereby directly applying a nitrification inhibitor to the urine patch. The PSM aucubin, found in *Plantago lanceolata*, was evaluated in this thesis.

The six experiments performed in this PhD research programme evaluated the potential for these proposed mitigation methods to alter urine patch N dynamics and reduce $N_2O$ emissions.

## 7.2 Summary of results and conclusions

### 7.2.1 Chapter 3: Non-urea ruminant urine nitrogen compounds: assessing their fate in a pasture soil and their impact on the urine patch nitrous oxide emission factor

Chapter 3 examined the fate of NUNCs in the urine patch in a laboratory experiment using $^{15}N$-labelling. This chapter also determined the effect of increasing the proportion of urine N excreted as NUNCs, rather than as urea, on urine patch $N_2O$ emissions in a field experiment. It was hypothesised that an increased proportion of urine N excreted as the NUNC allantoin, and its precursor NUNCs (hypo)xanthine and uric acid, would decrease the urine patch EF, due their potential to stimulate plant growth, and therefore plant N uptake of applied urine N.

The use of $^{15}N$ isotope clearly demonstrated that the lifetime of the NUNCs, creatine and hypoxanthine, in the soil was relatively short, with both NUNCs significantly contributing to both the soil N and plant N pools within 48 hours. Approximately 90% of applied NUNC-N in both treatments was available as $NH_4^+$ within 72 hours. It was concluded from the results of the laboratory trial that

NUNCs degraded rapidly in soils, and that urine N excreted as NUNC-N would contribute to soil $NH_4^+$-N, the substrate for nitrification (and further denitrification) and $N_2O$ emissions, similar to urea-N.

When examined under field conditions, increasing the proportion of urine N excreted as NUNCs did not alter the urine patch EF. Contrary to the hypothesis, plant N uptake was not stimulated when the proportion of urine N excreted as the PDs allantoin, uric acid, or (hypo)xanthine was increased. Thus, it was concluded that these compounds degrade rapidly in soils and likely contribute to soil labile N pools similarly to urea-N.

The results of the field trial led me to conclude that increasing the proportion of urine N excreted as NUNC-N, rather than as urea-N, does not alter urine patch N dynamics or $N_2O$ emissions, and therefore should not be further examined as an $N_2O$ mitigation technique. However, if grazing management strategies, which reduce total urinary-N excretion, find that the decrease in urine N is predominantly due to a decrease in urea-N, rather than NUNC-N, then the relative size of the NUNC-N pool, compared to the urea-N pool, would be increased. Under these conditions, it may be warranted to re-evaluate the effects of increasing the proportion of urine N excreted as PDs (allantoin, uric acid, or (hypo)xanthine), since these relative proportions would be higher than those used in this study.

However, as for now, this mitigation method does not currently show potential to significantly alter urine patch $N_2O$ emissions, it is not included in the following general discussion and future research recommendations.

### 7.2.2 Chapter 4: Potential inhibition of urine patch nitrous oxide emissions by *Plantago lanceolata* and its metabolite aucubin

This chapter presented two experiments, one a laboratory trial and one a field trial, which evaluated two objectives:

1. Determine if plantain contained a compound that inhibited nitrification and $N_2O$ emissions under urine patch conditions, and if so, determine if aucubin was the PSM responsible; and

2. Assess whether aucubin was present in plantain pasture soil and whether plantain pasture soil showed reduced nitrification and $N_2O$ emissions under urine patch conditions relative to a PR-WC pasture.

#### *7.2.2.1 Objective 1*

A laboratory trial was used to evaluate the effects of either a plantain leaf extract (PLE), which contained all extractable compounds in plantain leaves (including aucubin), or the pure chemical aucubin, applied as an aucubin solution (AS), on soil N dynamics and $N_2O$ emissions in urine-affected soils. The efficacy of both the PLE and AS were evaluated when applied in either urine or a urea solution to determine whether the variety of compounds found in ruminant urine had any effect on the potential inhibitory activity of the PSMs found in the PLE and AS.

A novel technique for aucubin extraction from soil was developed, and soil extractions showed that aucubin degraded rapidly in soils, with a residence time of approximately 4 days. The overall average $N_2O$ flux was significantly reduced in the Urea + PLE and Urea + AS treatments, when compared to the Urine treatment. Additionally, soil $NO_3^-$ concentrations on Day 29 were significantly lower in the Urea + AS, Urine + AS, and Urine + PLE treatments, compared to the Urine treatment. The similar significant reductions in soil $N_2O$ flux and soil $NO_3^-$ concentrations when either the PLE or AS was added to soils, indicated that plantain leaves contained a compound which inhibited nitrification, and that this chemical was aucubin. Furthermore, it appeared that these chemicals were active in either urine or a urea solution, since there were significant reductions in soil $NO_3^-$ concentrations when the PLE or AS were added to either urine or a urea solution. However, caution is needed when considering these results since it was apparent from the control treatments that the soil inorganic N dynamics and $N_2O$ fluxes in the laboratory trial were affected by $NH_3$ toxicity that occurred during the experimental period, which slows the nitrification process.

Thus, Objective 1 was re-examined by monitoring soil N dynamics and soil $N_2O$ flux after adding PLE and AS into the urine patch *in situ*. The additions of PLE and AS reduced the urine patch EF by 50 and 70%, respectively, when compared to the Urine EF. These results are similar to the laboratory trial, where adding either PLE or AS to urea significantly reduced average $N_2O$ flux. However, due to high variability in the field trial Urine treatment $N_2O$ flux measurements, only the 70% reduction in the Urine + AS treatment was statistically significant.

Soil analyses showed that under field conditions, aucubin was completely degraded within 24 hours, which was much shorter than the residence time found in the laboratory experiment, possibly due to the lower aucubin application rate, or differences in the soil pH (see 4.4.2). Furthermore, contrary to the results of the laboratory trial, soil $NO_3^-$ concentrations were not reduced when PLE or AS was added to urine, likely due to the aforementioned reasons for aucubin degrading faster under field conditions.

In conclusion, the efficacy of aucubin as a nitrification inhibitor was not clear from the results of these two experiments. While soil $N_2O$ emissions were reduced when PLE or AS was added to urine, these reductions were not consistently correlated with reductions in soil $NO_3^-$ accumulation. Chapters 5 and 6 were performed to gain further understanding of aucubin as a potential nitrification inhibitor.

#### *7.2.2.2 Objective 2*

Objective 2 was examined by monitoring soil N dynamics and soil $N_2O$ fluxes after adding a standard PR-WC urine to either a PR-WC or a plantain pasture. While statistical analyses could not be performed on this data, as explained in 4.2.2.5, no marked differences in soil N dynamics or $N_2O$ emissions were observed under urine patch conditions, regardless of pasture type. However, background $N_2O$ emissions (from control plots) were found to be lower in the plantain pasture, leading to the calculated "paddock-scale emissions after a grazing event" to be only 7.8 µg $N_2O$-N $m^{-2}$ $hr^{-1}$ in the plantain pasture, while PR-WC paddock-scale emissions were calculated to be 17.6 µg $N_2O$-N $m^{-2}$ $hr^{-1}$ (4.4.3).

It was concluded that plantain root exudation of aucubin may be low in fertilised pasture soils, since root excretion of PSMs that inhibit nitrification is typical of low N systems where plants need to increase N use efficiency and minimise N loss (Rice & Pancholy 1972; Subbarao et al. 2006). This conclusion was supported by the fact that no aucubin (detection limit of 0.001 mg $g^{-1}$ soil) could be extracted from plantain pasture soil during the experiment.

### 7.2.3 Chapter 5: Reassessing the potential for aucubin to reduce urine patch $NO_3^-$ accumulation and $N_2O$ emissions under field conditions

In this chapter, the efficacy of aucubin as a nitrification inhibitor was re-evaluated under field conditions. Three key changes were made, in comparison to the Chapter 4 field trial, to reduce variability between sampling chambers: the size of chamber bases was increased, the number of treatment replicates were increased, and the trial was performed on a site maintained with mowing, rather than grazing, to avoid background effects of previous urine patches. Additionally, the urine N loading rate was decreased to 500 kg N $ha^{-1}$, as this rate has been identified as more typical of a urine patch N loading rate from cows grazing plantain (Di et al. 2016). A similar reduction in the urine patch EF, compared to the Chapter 4 field trial, occurred during this experiment when aucubin was added to urine, but the difference between the Urine EF and Urine + Aucubin EF was not statistically significant. However, differences in soil $NO_3^-$ concentrations and soil surface pH indicate that

nitrification inhibition occurred when aucubin was added in urine from Days 4-7. It is known that the aglycone of aucubin, aucubigenin, is the active degradation product of aucubin that likely inhibits nitrification (Bartholomaeus & Ahokas 1995). It is likely that aucubin degrades rapidly in soil to form aucubigenin, which then actively inhibits nitrification for a short period.

Therefore, it was concluded that aucubigenin, the aglycone of aucubin, actively inhibited nitrification from Days 4-7, leading to a reduced accumulation of soil $NO_3^-$ and a delayed decrease in soil surface pH. However, the inhibitory capacity of aucubin at this rate of application was not high enough to produce an overall significant reduction in urine patch $N_2O$ emissions. Since this aucubin application rate corresponded to only 10% of the highest calculated potential aucubin excretion rate, calculated in 4.4.4. Thus, an additional experiment to determine the effects of increasing aucubin application rate on urine patch $N_2O$ emissions was warranted. This experiment was performed in Chapter 6.

### 7.2.4 Chapter 6: Urine patch $N_2O$ emissions under varying rates of aucubin application

Three rates of aucubin application (A1: 47 kg $ha^{-1}$; A2: 243 kg $ha^{-1}$; and A3: 487 kg $ha^{-1}$) were added to urine and assessed for their impacts on soil N dynamics and $N_2O$ emissions in a laboratory trial. These rates represent the rate used in the previous field trials (based on Dietz et al. (2013); 47 kg $ha^{-1}$), half the highest calculated potential aucubin excretion rate (from 4.4.4; 243 kg $ha^{-1}$), and the highest calculated potential aucubin excretion rate (from 4.4.4; 486 kg $ha^{-1}$). It was hypothesised that increasing the aucubin application rate would lead to an increased reduction in $N_2O$ emissions and $NO_3^-$ accumulation.

The average flux from Days 5-11 was significantly lower in the Urine + A2 and Urine + A3 treatments than in the Urine treatment, but the overall average flux for the full measurement period (37 days) was not different between all urine-amended treatments. Aucubin contains a glucose ring, which is removed when aucubin is degraded to aucubigenin, and this added glucose-C may have stimulated microbial activity. This stimulation of microbial activity may have offset the expected decrease in $N_2O$ emissions from aucubin and/or aucubigenin's inhibitory effect on nitrification.

Similar to Chapters 4 and 5, there was a period (Days 7-17) in the Urine + A2 and Urine + A3 treatments where soil N and soil pH measurements indicate that nitrification was inhibited by aucubin. This also corresponds to the period where lower $N_2O$ fluxes were measured in these treatments. However, similar to Chapters 4 and 5, this short period of inhibitory activity was not sufficient to significantly reduce overall average $N_2O$ flux during the 37 day experiment. Therefore, it was concluded that livestock consumption of plantain, and subsequent excretion of aucubin into the urine patch, could reduce urine patch $N_2O$ emissions, but the percentage of plantain in the diet, and

percent excretion of ingested aucubin, would both need to be almost 100% to produce a significant effect. However, the combined effects of aucubin excreted in urine onto plantain pasture soil, which may contain aucubin from plantain root exudation, were not evaluated in this thesis and may increase the significance of nitrification inhibition.

## 7.3 General conclusions - aucubin as a nitrification inhibitor

The effectiveness of a novel biological nitrification inhibitor, aucubin, was evaluated in four experiments. However, in these experiments, the addition of aucubin to bovine urine did not consistently produce a significant reduction in urine patch $N_2O$ emissions. A consistent trend was found between experiments (Chapters 4-6), where a short period of nitrification inhibition appeared to occur approximately 4-10 days after urine application. It was hypothesised that this period of inhibition corresponds to the period where the active aglycone of aucubin, aucubigenin, was present in soils. However, this period of inhibitory activity was not sufficient to produce statistically significant reductions in overall average urine patch $N_2O$ emissions, or the urine patch EF, over a longer term.

From the results of these experiments, it was apparent that the application of aucubin in urine to soil is not as effective as the application of synthetic nitrification inhibitors previously identified, such as DCD, which can reduce urine patch $N_2O$ emissions by up to 70% (Di & Cameron 2002a; Di et al. 2007). However, other recent research found significant and longer lasting BNI effects from plantain growing in the soil (Carlton 2017; Luo et al. 2018), which may provide a continual source of aucubin, via root exudation. These studies did not determine the mechanism behind this reduction, but it likely could be due to the inhibitory effects of aucubin on nitrification. While this effect was not observed in our research (Chapter 4, Objective 2), plantain root exudation of aucubin, the derivatives of aucubin (namely aucubigenin), or other BNI chemicals, is key topic for future research, as this would provide a continual source of active BNI chemicals in soils, rather than a single application in urine. Furthermore, it is possible that the combined effects of urinary aucubin excretion and continual plantain pasture aucubin root exudation could increase the degree of nitrification inhibition in the urine patch.Further research of plantain, and its embodied compound aucubin, as a nitrification inhibitor is warranted, due to the observed nitrification inhibition activity in the aforementioned studies, and presented in this thesis.

The focus of the experiments performed for this thesis was to evaluate the effects of the two proposed mitigation methods on urine patch N dynamics and $N_2O$ emissions, with the assumption that these altered urine compositions (e.g. varying NUNC-N concentrations and excretion of PSMs in urine) are possible. However, future work needs to evaluate all levels of the grazing management

system (e.g. quantity and seasonality of PSM concentrations in leaf dry matter, survival and potential transformation of PSMs during transport through the rumen digestive system, rate and composition of urine-embodied PSMs). This study was one of many parallel ongoing investigations, with studies performed by others examining the effects of ruminant urine chemistry and soil microbial biology.

## 7.4 Recommendations for future research

The key recommendations for future research are:

1. Determine the rate of aucubin, or the derivatives of aucubin, excreted in urine from ruminant livestock grazing various percentages of plantain in their diet. The aucubin application rates used in the experiments reported in this PhD were based on a study that was not performed under urine patch conditions (Dietz et al. 2013), but were based on calculated potential aucubin excretion rates. Direct quantification of aucubin excretion rates would allow for future experiments to base experimental aucubin application rates on true measured values.

2. After determining the rate of aucubin, or its derivatives, excreted in urine, it is necessary to track the fate of these compounds in the urine patch, potentially by using isotope tracing methods. Particular focus should be paid to the active aglycone of aucubin, aucubigenin, and its ability to cross-link with proteins and/or enzymes associated with nitrifying bacteria.

3. Assess the impact of soil conditions (e.g. soil carbon content, soil moisture content, etc.) on the fate of aucubin, or its derivatives, in soil and the efficacy of these compounds as nitrification inhibitors.

4. Perform soil microbial analyses, particularly focussing on the population and activity dynamics of AOB, to determine the degree of nitrification inhibition under varying rates of aucubin application.

5. Explore the multiplicative effects of adding urine containing aucubin onto plantain pasture, where plants may also exude aucubin, via root exudation, into the pasture soil. Chapter 4 analysed these two effects separately, but under realistic conditions, grazing livestock would consume aucubin in plantain forage, and then excrete aucubin in their urine back onto the plantain pasture that they were grazing.

6. Set up a simulation of persistent root release of aucubin and/or decomposition of residual herbage to determine total plant inputs of aucubin into the soil.

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